University of Alberta

Natural Antifungals:

Screening, Isolation, Synthesis, and Mechanism of Action

by

Soroush Sardari Lodriche ©

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The undersigned certify that they have read, and recommend to the faculty of Graduate Studies and Research, for acceptance, a thesis entitled **NATURAL ANTIFUNGALS: SCREENING, ISOLATION, SYNTHESIS, AND MECHANISM OF ACTION** submitted by Soroush Sardari L. in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences.

Dr. Mohsen Daneshtalab (supervisor)

Dr. Ronald G. Micetich (co-supervisor)

Dr. Franco Pasutto (co-supervisor)

Dr. Edward E. Knaus (committee chair)

Dr. Randy S. Currah (committee member)

Dr. Hsing-Jang Liu (committee member)

Dr. Sansei Nishibe (external examiner)

April 9, 1998

Dedicated to

God

Parents

Mentors

Friends and Siblings

Nature

Science

Religion

Philosophy

Abstract

Crude extracts from 40 Iranian and Canadian plants were tested for their antifungal activity against several species of *Aspergillus, Candida,* and *Cryptococcus*. The extracts of *Diplotaenia damavandica, Heracleum persicum, Sanguisorba minor,* and *Zataria multiflora* exhibited the widest spectrum of activity. Cytotoxicity tests in KB cells were performed on some of the most promising plant extracts. *D. damavandica* and *Bunium persicum* were found to exhibit the lowest cytotoxicity among the plants studied.

Bioassay guided isolation of *D. damavandica* active constituents led to identification of four coumarin compounds, angelicin, libanorin, psoralene, and auraptene, which showed antifungal activity. The cytotoxicity evaluation of the fractions demonstrates almost no toxicity.

Fraxinus americana has been used traditionally among native Indians of North America for different purposes. Four compounds, verbascoside, 10-hydroxyligstroside, ligstroside, and syringin, were identified in the bark of *F. americana*. Ligstroside showed weak activity. The presence of phenolic phenylpropanoids can justify the traditional use of the bark of this plant especially for inflammatory conditions.

Several derivatives of angelicin were designed and synthesized. The correlation of structure and activity as well as the relevance of the lipophilicity factor, log $D_{7.0}$, to the antifungal activity is discussed. Angelicin and several potent antifungals proved to be non-toxic in human cell line cytotoxicity assay.

It was found that angelicin and db-cAMP increased the cellular level of ergosterol in treated *C. albicans*. This evidence supports the idea that coumarins do not act like azole antifungals. The interaction studies between cAMP derivative and different antifungal

drugs suggested the existence of antagonism, which was observed in a similar fashion between the azole drugs and phosphodiesterase inhibitors. This finding suggested a role for cAMP in ergosterol metabolism.

Indirect evidence was found in relation to the action of coumarins on the pH controlling system in fungi. The coumarins showed synergism with most of the agents acting on proton pump ATPase. Intracellular pH was reduced in the treated fungi as well. Morphological studies with the electron microscope revealed multiple budding in coumarin treated *Candida* cells which could be attributed to the phosphodiesterase inhibitory activity of coumarins. The reason for the observed changes in the vacuolar systems of the fungal cells is not quite clear.

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Glossary of Abbreviations

µg	microgram(s)
µl	microliter(s)
µM	micromolar(s)
λ_{max}	wavelength of absorption maximum, in UV spectra
$[\alpha]^t_\lambda$	specific rotation at t°C at a given wavelength, λ, in nanometers
A	absorption
A. niger	*Aspergillus niger*
Ac	acetyl
AcOH	acetic acid
Ac_2O	acetic anhydride
AC	adenylate cyclase
ACI	adenylate cyclase inhibitor
ADP	adenosine diphosphate
AmB	amphotericin B
AMP	adenosine monophosphate
amu	atomic mass unit
AP	aerial part
aq	aqueous
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
ATCC	American Type Culture Collection
B	bark
bp	boiling point
Bu	butyl
C. albicans	*Candida albicans*
cAMP	cyclic 3', 5' adenosine monophosphate
ca.	(*circa*) about
Cald	calculated
$CDCl_3$	deuteriochloroform
CFU	colony forming unit
conc.	Concentrated
D	dextro
d4-MeOH	tetradeuteriomethanol
d6-DMSO	hexadeuteriodimethysulphoxide
DCC	dicyclohexyl carbodiimide
DCCD	dicyclohexyl carbodiimide
DMF	dimethylformamide
DMSO	dimethylsulfoxide
dpm	decay per minute
E	entgegen
ES-MS	electrospray mass spectroscopy
Et	ethyl
FAB-MS	fast atom bombardment mass spectroscopy
5-FC	5-fluorocytosine
FIC	fractional inhibitory concentration
g	gram(s)
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
Glu	glucose

h	hour(s)
HRES-MS	high resolution electrospray mass spectroscopy
HR-MS	high resolution mass spectroscopy
HPLC	high performance liquid chromatography
i.d.	inside diameter
IBMX	(3-Isobutyl)-1-methylxanthin
IC	inhibitory concentration
IR	infrared
L	levo
L.	(Carolus Linnaeus) Carl Linne (1707-1778)
M	molar
m/z	mass to charge ratio
Me	methyl
mg	milligram(s)
MFC	minimum fungicidal concentration
MIC	minimum inhibitory concentration
min	minutes
ml	milliliter(s)
mM	millimolar(s)
mp	melting point
MS	mass spectroscopy
msec	millisecond
NCCLS	National Committee on Clinical Laboratory Standards
NDA	new drug application
nm	nanometer(s)
NMR	nuclear magnetic resonance
°C	degrees Celsius
PA	Pascal (pressure unit)
PDI	phosphodiesterase inhibitor
pH	power of hydrogen, acidity where pH = log (1/[H^+])
PLM	Provincial Laboratory Micro-collection (Edmonton, Alberta)
PPA	polyphosphoric acid
ppm	parts per million of chemical shifts (δ) from tetramethylsilane in NMR spectra
Pr	propyl
R	*rectus*
R	root
rpm	revolution per minute
RPMI	synthetic cell culture medium
Rt	retention time
S	seed
S	*sinister*
S. cerevisiae	*Saccharomyces cerevisiae*
SAR	structure activity relationship
SDA	Sabouraud dextrose agar, a complex culture medium
SE	standard error
SEM	scanning electron microscopy
sp., spp.	species, singular and plural
TEM	transmission electron microscopy
TLC	thin layer chromatography
TMS	trimethylsilyl
UV	ultraviolet
var.	variety
Z	zusammen

Chapter 1

Introduction and Background

Use of phytomedicine dates back to the beginning of civilization and still prevails in most of the world's population (approximately 80% routinely use traditional medicine or folklore medical practices). In North America, a quarter of all prescriptions are from plant products or derivatives, and 75 percent of these follow traditional uses by native cultures (Balandrin, *et al.*, 1993; Farnsworth, 1996). This figure is quite high considering that patent laws also discourage the development of plant products as pharmaceuticals by barring patent exclusively for phytochemicals that had ever appeared in the scientific literature.

Analysis of the number and sources of anticancer and anti-infective agents indicates that over 60% of the approved drugs and pre-NDA (New Drug Application) candidates for the period of 1989-1995, excluding biologics, are of natural origin (Cragg, *et al.*, 1997). The data related to the source of compounds in all therapeutic categories and anti-infectives are depicted in Figure 1-1. Nature is a rich source of diversity and variation. As can be seen about half of the compounds in all categories originate from nature. The number of substances utilized to treat infectious disease increases dramatically for the natural and semisynthetic group. This contribution of natural products is very minute considering the real potential and diversity that exists in nature. It has been estimated that only 5-15% of the approximately 250,000 species of higher plants has been systematically investigated for the presence of bioactive compounds (Balandrin, *et al.*, 1993). Therefore, it is quite justifiable to expect more novelty and contribution from natural sources.

Early pharmacognosists were legitimately concerned with extracting the active compound from the natural source. In this regard, simple bioassay-guided isolation/fractionation almost invariably led to the isolation of the active constituents. The

concern of finding the active constituent may sometimes interfere with the urge for searching for novel compounds in plant extracts. The modern technology of MS-MS and HPLC coupled with different detectors like UV, MS, and recently NMR (Lindon, *et al.*, 1996) helps the scientist to shortcut the way to identification of novel constituents in an active fraction. After all, luck is still an important player in this field.

To understand the precise mechanism involved, contemporary pharmaceutical research uses designed chemicals, which are aimed at very specific targets. While this approach occasionally results in successful new medication, it is limited to mechanisms that we already know and understand. Progress is most often an incremental improvement to an existing medicine, such as development of β-lactam antibiotics, reuptake inhibitor antidepressants, or azole antifungals. A receptor or a group of compounds has a limited capacity for amelioration to act as a therapeutic site of action. Investigation of purported medicinal activity of natural sources stands a good chance of leading to new mechanisms and novel treatments.

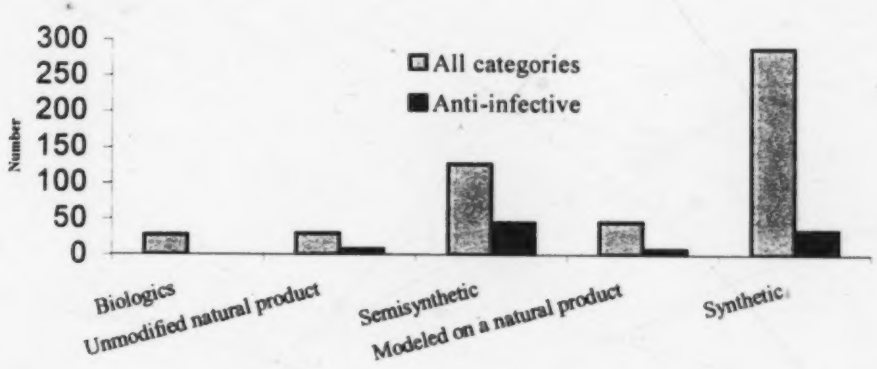


Figure 1-1. New approved drug sources: 1983-1994 (Cragg, *et al.*, 1997).

Infectious diseases are potentially the biggest threat to human safety in the post-cold war era.

Historically, although dermatophytes are among the most common agents of diseases in man and other animals, fungal infections in human generally have had less impact on

mankind than bacterial, viral infections and plant fungal infection. There are no great pandemics, no equivalents to the plagues, potato famine, yellow fever, and cholera that have caused such devastation (Hunter, 1995). The last decade has seen an increase in both occurrence of fungal infections and shift of focus point. Although many of the dermatophytes are now under control, other fungal infections have become more common.

With the employment of modern chemotherapeutic procedures in the 1960's, susceptible hosts were created for several opportunistic organisms and with the emergence of the AIDS era in the 1980's, a broad range of opportunistic pathogenic fungi are making their appearance on the medical scene. The role of commensals, such as *Candida* species, is changing. Ranging from a 75% increase in small hospitals to over 400% increase in some large care centers, *Candida* is now ranked as the third most common causative agent of nosocomial blood stream infections in most hospitals (Pfaller, *et al.*, 1992).

A detailed presentation about all the human pathogenic fungi could take volumes of books. However, a short discussion about the species that represent the most important fungi causing infection in human at the present time is as follows (Calvet, *et al.*, 1996):

The species of *Candida* produce 4-6 μm diameter cells that normally colonize the gastrointestinal and lower female genital tract of humans. *Candida albicans* germinates rapidly under physiological conditions and forms a long thin projection called a germ tube, which may be an important virulence factor. Other species of *Candida* do not form germ tubes as readily, allowing for rapid differentiation of *albicans* from non-*albicans* species *in vitro*. *C. albicans* is the most common species causing human disease, but other pathogenic species include *C. tropicalis, C. guillermondii, C. krusei*, and *C. pseudotropicalis*. *Torulopsis glabrata*, once classified as a *Candida* species, is often included in the group causing candidiasis. The incidence of *Candida* vaginitis may be as high as 20% among females of reproductive age and approximately 75% of all women experience an acute infection during their life span (Monk, *et al.*, 1994).

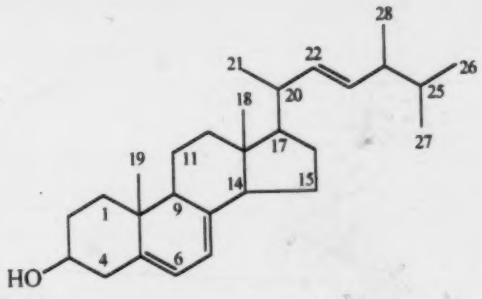
Aspergillus species are found throughout the world with ubiquitous distribution in the environment. These fungi form hyphae 2-4 μm wide, which are septate and branched at an acute angle to the main stem. The fungus grows well on many kinds of organic material. *Aspergillus* spores are found throughout hospitals, in air samples, dust and environment cultures. Exposure to the spores through the respiratory tract is a common occurrence, yet *Aspergillus* species are not a common cause of human disease except in the severely immunocompromised population. Among the many species of *Aspergillus*, causing invasive disease, *A. fumigatus, A. flavus*, and *A. niger* are the most common.

Cryptococcosis is caused by an encapsulated yeast measuring approximately 4-6 μm in diameter. The capsule varies in size depending on growth conditions. In general, the fungus tends to be sparsely encapsulated in nature and highly encapsulated in host tissues. Based on the different serotypes, the organism is classified into two main groups, *C. neoformans* and *C. gattii*. These two differ in geographical distribution. Most of the clinical isolates in the U.S. are *C. neoformans*. The most common organ affected by dissemination is the CNS with meningoencephalitis or meningitis presentations. *Cryptococcus* has a worldwide distribution, found in soil and especially in pigeon droppings.

Before discussing antifungal drugs, it would be suitable to mention potential fungal cell targets for development of different agents combating mycoses. Since the fundamental organization of all eukaryotes is essentially similar, the targets available that differ sufficiently between fungi and mammalian cells are fewer than between prokaryotes and the mammalian system. Thus many interesting agents prove too toxic for systemic use. However, there is a steady effort in the scientific community to understand the basic structures specific to fungi and use them as the potential targets for antifungal therapy. In the following paragraphs, these focal points have been briefly reviewed.

Fungi are among the most primitive organisms that synthesize sterols, principally ergosterol. The functions and synthesis of sterols have been effective targets for fungal control in different areas including pharmaceutical and agricultural applications.

Ergosterol biosynthesis is dependent on molecular oxygen and haem components that are synthesized aerobically (Parks, *et al.*, 1995). At least nine subsequent transformations are required to synthesize ergosterol, the ultimate end product of the isoprenoid pathway.



The structure of ergosterol

The main steps in the biosynthesis of ergosterol are shown in Figure 1-2 (Baldwin, *et al.*, 1995). The complexity of the biosynthetic pathway is evident in the whole process. It can be concluded that because of the high energy cost of this biosynthetic pathway to the organism, ergosterol must be an essential cellular component for it to have emerged and persisted in the evolutionary process.

Another target point for antifungal action is the fungal cell wall. The fungal cell wall is a complex multilayered structure with a high polysaccharide content, the composition of which varies between species. Detailed knowledge of this structure is lacking for many fungi of medical importance. The bulk of information available is on *C. albicans* and *S. cerevisiae* cell walls. The fungi of clinical interest all contain variously linked glucans [glucose polymers linked through α- and β-(1,3) or (1,6) linkages], chitin (repeating *N*-acetyl glucosamine) and mannoproteins. The agents active on the cell wall of fungi have been used in agrochemical industry for many years e.g., polyoxins. There are 1,3-β-glucan synthesis inhibitors, such as echinocandines, and pneumocandins, as the center of many studies for human usage. A chitin synthase inhibitor, nikkomycin Z, is in phase I clinical trials. However, to date, no such compound has reached the market for medical use. Lack of activity against *Cryptococcus*, limitation in the spectrum of activity to

organisms rich in β-glucan, and higher concentrations needed to inhibit zoopathogenic fungi rather than phytopathogens are among the factors limiting their use in medicine (Hunter, 1995).

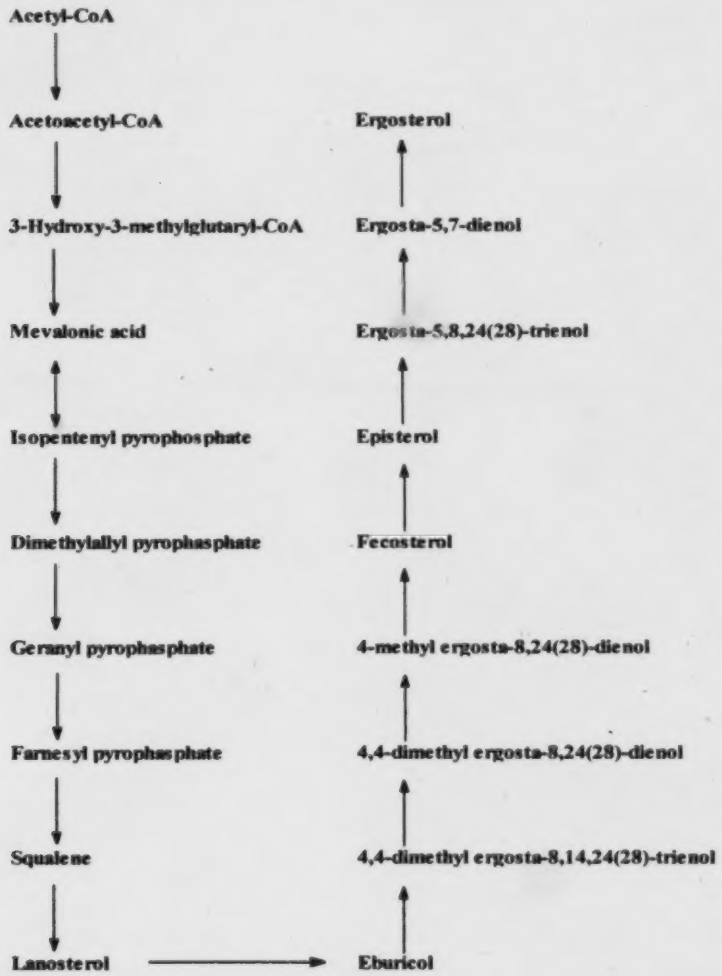


Figure 1-2. The main steps in the ergosterol biosynthetic pathway.

Tubulin is yet another site for the antifungal activity of several agents like griseofulvin. Tubulins form part of the polymeric protein complexes known as micotubules, which are found in all eukaryotic cells (Butters, *et al.*, 1995).

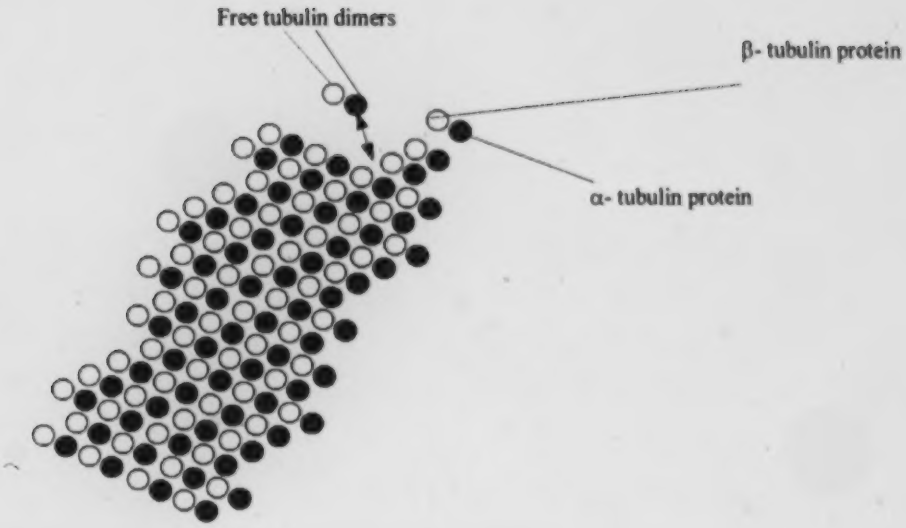


Figure 1-3. Structure of tubulin assembly.

Heterodimers between α- and β-tubulin proteins are condensed into tubulin elements (MWt = 100,000) which form the core of these microtubules (Figure 1-3). Growing tubulin filaments are stabilized through the binding of various microtubule accessory proteins, which determine the function of the particular microtubules. Microtubules are dynamic structures where the balance between GTP-dependent assembly of the tubulin heterodimers and their hydrolysis determines whether microtubules grow or not (Figure 1-3). Microtubules form part of the cytoskeleton. They not only provide passive support for the cell, but also play key roles in the trafficking of cellular organelles. They also form the spindle, which guides movement of chromosomes during cell division.

The plasma membrane proton pump H^+-ATPase is an important new target for therapeutic intervention. The H^+-ATPase is a predominant membrane protein that belongs to the P-type (plasma membrane-type *vs* V-type, or vacuolar type) ATPase family of ion translocating ATPases (Seto-Young, *et al.*, 1997). This essential enzyme plays a critical role in fungal cell physiology by maintaining the large transmembrane

electrochemical proton gradient necessary for nutrient uptake and by regulating intracellular pH (Monk, *et al.*, 1995). It is one of the few antifungal targets which have been demonstrated to be essential by gene disruption techniques (Venema, *et al.*, 1995; Morii, *et al.*, 1993). In addition to its role in cell growth, the H^+-ATPase has been implicated in fungal pathogenicity through its effect on dimorphism, nutrition uptake, and medium acidification (Slayman, 1990). The family of P-type ion pumps include Na^+, K^+-ATPase and H^+, K^+-ATPase, which are molecular targets for several clinically important therapeutics like digoxin and omeprazole (Monk, *et al.*, 1994). These drugs are enzyme specific antagonists, which inhibit via an interaction with the extracellular surface of their respective target enzyme. Therefore it would be possible to develop similar types of antagonists specific for the fungal H^+-ATPase. Highly specific H^+-ATPase antagonists capable of acting from outside the cell should be extremely valuable as antifungals. This is especially pertinent given the increasing clinical problem of drug resistance due to multi-drug resistance pumps. Extracellularly directed reagents do not cross the cellular membrane, and therefore are not substrates for these high capacity drug pumps (Seto-Young, *et al.*, 1997; Raymond, *et al.*, 1992).

DNA topoisomerases, a class of enzymes that change the topological structure of DNA, have been shown to be the target of many therapeutic agents, including antibacterial agents (quinolones) and anticancer agents. These drugs inhibit the enzyme in a unique way so that the enzyme is converted into a cellular poison (Shen, *et al.*, 1992). Etoposide, an inhibitor of mammalian topoisomerase II, is inactive against the yeast enzyme, although good inhibition is shown by amiloride and novobiocin (Figgitt, *et al.*, 1989; Shen, *et al.*, 1994). Fungal topoisomerase I has been purified (Goldman, *et al.*, 1997), and has shown resistance to monobenzimidazoles, protoberberines and nitidines (Goldman, *et al.*, 1997; Fostel, *et al.*, 1996). These results suggest that the nuclear fungal topoisomerase I may be sufficiently different from its human counterpart to serve as a molecular target for the development of antifungal drugs.

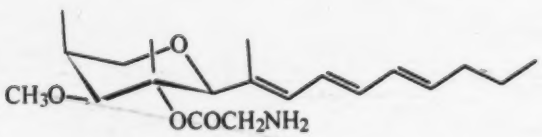
Agents to treat systemic mycoses were few until the advent of the modern chemotherapeutic era. Oral potassium iodide has been used for treating subcutaneous

sporotrichosis for several decades and has remained the drug of choice until very recently. The 1950s and 1960s saw the discovery of a number of specific and highly active antifungal agents both natural and synthetic compounds, many of which remain in use today. The main agents that are currently used are:

Amphotericin B (AmB): A polyene discovered in 1956, produced by *Streptomyces nodosus*. The first effective compound available for systemic mycosis, used against serious mycotic infections. AmB is regarded by many as the drug of choice for the seriously ill patients, in spite of a number of major drawbacks. It is nephrotoxic, poorly tolerated, and has such low solubility in biological fluids that it has to be formulated as a colloidal suspension in bile salts and administered as a slow infusion (Sarba, 1990). In addition, to reduce the toxicity, lipid-based formulations of AmB (mostly liposomal formulations) have been either marketed or are undergoing further studies (Hiemenz, *et al.*, 1996; Wasan, *et al.*, 1997). Nevertheless, it is still claimed by many to be the best standard, against which other agents should be compared. It has a broad spectrum of activity and is fungicidal against most species, at least *in vitro*. The newer azoles have still not replaced it for many indications, particularly in the immunocompromised host. The polyenes exert their effect by associating with the eukaryotic membrane sterols and disrupting membrane integrity, as can be shown by a rapid efflux of potassium ions from treated cells (Hunter, 1995). It is believed that AmB inserts into membrane by having a high affinity for ergosterol in the fungal membrane. This affinity is lower for cholesterol, therefore, AmB shows a greater effect on fungi in low concentration than on host cells.

Azoles: These are synthetic broad-spectrum agents, many of them only available for topical use. Miconazole and clotrimazole were first described in 1969. Ketoconazole, first described in 1979, was the first azole to have significant oral activity, but it had limited value for the treatment of serious systemic fungal infections (Hunter, 1995). The advent of triazoles marked a turning point for these compounds of which two have been considered important in clinics; fluconazole, which is also available in injectable form, and itraconazole. Both have undergone extensive clinical studies and have proved to be valuable drugs. Although fluconazole shows a very significant efficacy against *C.*

albicans, and *Cryptococcus*, it is not effective against *Aspergillus*. Itraconazole has shown a remarkable efficacy against a wide range of fungal infections including Aspergillosis (Bailey, 1990). Several triazoles have been described in recent years that are claimed to have advantages over fluconazole and itraconazole, but mostly have either toxicological or formulation problems. A natural product Ro 09-1470, which has been isolated from *Penicillium* species and several strains of *Aspergillus sclerotiorum*, was found to inhibit lanosterol C-14 demethylase in the same way that azoles do (Aoki, *et al.*, 1992; Matsukuma *et al.*, 1992). Although this compound is not potent enough for clinical use, it is the first natural product to be reported as having such a mode of action.



Ro 09-1470

Allylamines: have antifungal effects against a broad spectrum of fungi including *Aspergillus* species, *C. neoformans* and some *Candida* species. The allylamines like naftifin and terbinafin are highly selective towards the fungal sterol biosynthetic pathway, having activity several orders of magnitude less toward mammalian cholesterol biosynthesis. These compounds are inhibitory to squalene epoxidase isolated from fungi like dermatophytes and *Candida* (Ryder, 1991).

Morpholines: have a broad spectrum of activity against dermatophytes, *Candida* species, *Cryptococcus*, and dimorphic fungi like *Histoplasma*. Fenpropimorph, is used as a topical antifungal. The mode of action of these compounds is inhibition of delta 8-7 isomerase and delta 14 reductase in the biosynthetic route of sterols (Mereer, 1991; Hunter, 1995). Sites of action of ergosterol biosynthetic pathway affectors are shown in Figure 1-4.

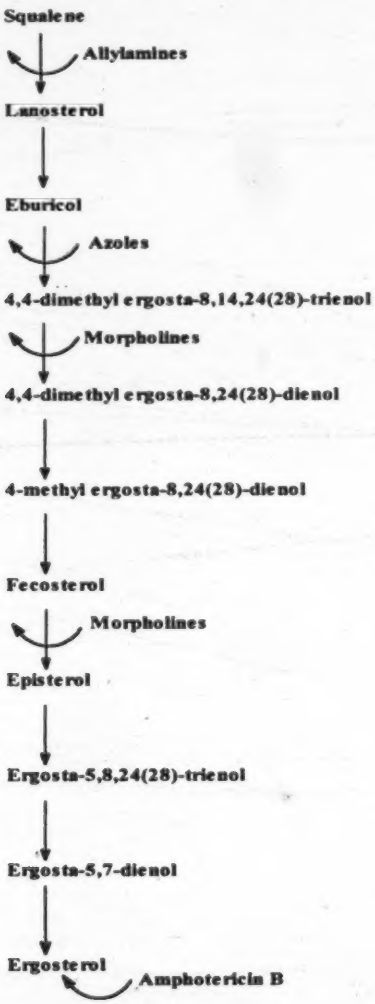


Figure 1-4. The main sites of actions of membrane active antifungal drugs that affect the ergosterol biosynthetic pathway (Parks, *et al.*, 1995).

Flucytosine (5-fluorocytosine, 5FC) is a synthetic antimetabolite whose antifungal properties were recognized in 1964. Flucytosine is a prodrug of 5-fluorouracil; it is taken into the fungal cell by a cytosine permease and deaminated to 5FU. The resulting 5-fluorouridine triphosphate replaces uracil in RNA and 5-fluorodeoxyuridylate inhibits thymidylate synthase, thus affecting DNA biosynthesis (Kerridge, 1986). It is active orally against *C. albicans* and *Cryptococcus neoformans* but the occurrence of resistant strains limits its value (Hunter, 1995).

Griseofulvin: is an oral antibiotic produced by *Penicillium* species (including *P. griseofulvum*), and has high activity against dermatophytes introduced in 1958. Griseofulvin interacts with tubulins acting as a mitotic poison (Kerridge, 1986). Griseofulvin causes a characteristic curling of hyphae, but also disrupts nuclear division in a way that suggests interference with microtubule function. It does not, however, bind to α- and β-tubulin protein, but interacts with microtubule associated proteins.

The main part of the research as described in this thesis is a study of the potential of natural products in the field of antifungals. In this respect, one of our earliest sections, chapter 2, deals with the selection and screening of the targeted sources against different fungi, as described above. Following that, the isolation and structure elucidation of the active constituents involved in the observed bioactivities was carried out. These will be discussed in chapters 3 and 4. Synthesis and further modifications of the obtained lead compounds are presented in detail in chapter 5. Chapter 6 deals with the observations leading to hypotheses regarding the mode of action of a few selected antifungal molecules. The summary and conclusions are compiled in the very last chapter (7).

The following reasons were the main factors for the initiation of this research. Since the actual drug development process is done mainly in pharmaceutical companies with considerable resources, the work that is presented here would be in fact a miniature compared to their practice. In addition to the educational aspect of the process, the multidisciplinary feature of this research exposes the candidate to a variety of expertise and an appreciation of the interdisciplinary nature of pharmaceutical research. This

exposure brings more insight of the discipline to the researcher, including the capability of coordination and team-work. I believe practicing the above profiles for 4-5 years would bring self-sufficiency and confidence to the person, which would be beneficial for his/her career.

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Chapter 2

Antifungal Activity of Selected Iranian and Canadian Plants

Introduction

The increasing demand in finding novel antifungal agents capable of overcoming deep-seated mycoses, and resistance induction has diverted scientists attention toward natural products. Echinocandins, Pneumocandins, Pradimycins, and Nikkomycins are representatives of novel classes of antifungals with novel mechanism of activity. These agents, which have been isolated as fermentation products, have been semi-synthetically modified to produce favorable pharmacokinetic profiles. The structural complexity and narrow spectrum of activity are the major draw-backs for the future development of these compounds. Since plants are considered as an important source of simple bioactive molecules, we have tried to systematically study the antifungal properties of selected plant species.

Regarding the diversity in the population of plants around the world, in this project we tried to focus mainly on the plants native to Iran and Canada. Iran has a rich flora of 6000-7000 species (World Conservation Monitoring Center, 1992) of which many are native and still unstudied for their phytochemistry or bioactivity. However, the strong ethnopharmaceutical literature of Iran provides us with valuable information on the widely utilized plant material in folk medicine. In this chapter, we wish to report the result of antifungal activity among 40 species of selected plants related to 17 families and 36 genera.

Materials and Methods

Micro-organisms and culture media

The following fungal species were used in this study:

Aspergillus fumigatus PLM 712, *Aspergillus niger* PLM 1140, *Candida albicans* 200/175, *Candida albicans* ATCC 14053, *Candida albicans* Y01-09, *Candida glabrata* ATCC 90030, *Candida guilliermondi* IF 00838, *Candida kefyr* ATCC 38296, *Candida*

krusei ATCC 44507, *Candida pseudotropicalis* Y06-01, *Candida tropicalis* ATCC 13803 and *Cryptococcus neoformans* KF-33. The micro-organisms were cultures obtained from American Type Culture Collection (ATCC), University of Alberta Hospital, kindly provided by Dr. A. Sekhon, and SynPhar Labs., Edmonton. Most of the fungi used in this study are human isolates. The fungi were grown and subcultured on Sabouraud dextrose agar (SDA) plates at 35^0C for 24-48 h and kept at 4^0C for not more than 3 days before preparation of the inoculum.

Plant Material

The plant material were collected from different locations in Iran and the city of Edmonton (Canada) (Table 2-1). The plants were identified by G. Amin and S. Sardari and voucher specimens were deposited at the herbarium, Faculty of Pharmacy, Tehran University of Medical Sciences. In all cases, the aerial part of the plant was used, unless otherwise specified. The plant material was air dried, and then ground.

To increase the chance of finding active extracts, a non-random screening program was performed on Iranian plants. However, the Canadian plants were mostly chosen at random. In this way, Iranian plants were selected based on the possession of one or more of the following characteristics:

1. Screening: A survey of the activity of plants against dermatophytes like Trichophyton served as a primary starting point (Salehi, *et al.*, 1993; Karamati, *et al.*, 1993).

2. A) Traditional Medicine: Different references regarding Iran's traditional medicine such as volume II of Canon, composed by Avicenna about 1000 years ago (Chishti, 1991; Gruner, 1930; Shah, 1966; Sharafkandi, 1991), and other relevant literature published by Amin (1991), and Hooper *et al.,* (1937) were consulted. B) Regarding plants that are used in other parts of the world for their speculative antifungal related activity, other references in this respect were reviewed (Altschul, 1973; Von Reis, *et al.,* 1982). In consulting such references, key words such as infection, fever, thrush, ringworm, and skin disease were used to indicate a possible correlation to antifungal activity. Other traditionally used medicinal plants were also considered in this category. Often the botanical names are not in complete agreement with the common names and therefore the assigned traditional use may not be accurate; this study attempted to include only those plants that have been matched with their complete botanical names.

3. Reports of bioactivity: Reports on any plant bioactivity that might or might not be related to the desired antifungal activity was another factor in this study.

4. Rarity: The plants with no or very few reports on their phytochemistry and/or biological activity had priority. This factor was considered to be very important and any plant that was in this category by itself or in combination with other factors, was included in the study.

5. Chemotaxonomy: A) There was a trend to choose plants belonging to the Compositae family due to the results reported by Hoffman *et al.,* (1993). In this report, after screening of 300 plants from 69 plant families, the most effective plants were found to be in the Compositae. The chemical diversity and an advanced phylogenetic state (Alston, *et al.,* 1963) might contribute to this result. B) A taxonomically close relative of a reported bioactive plant is put in this category.

6. Food or spice: Safe usage of plants as food or spice, traditionally utilized by people who live in Iran, was considered as a positive factor.

7. Availability.

8. Ecology: Any known or reported case of interaction, of the plant with livestock, human or other living organisms including fungi, was considered.

To increase the chance of hitting novel cases and reduce the possibility of redundancy in our work, only plants with no scientific report of antifungal activity in general or against the species tested, were selected.

Preparation of the crude extracts

The plant powders were extracted at room temperature by percolation with MeOH. The extracts were concentrated under reduced pressure. The condensed products were weighed and kept at 4^0C prior to the test.

Antifungal Tests

Agar dilution: The method used for this test was adopted from Muanza *et al.*, (1994), and Mitscher *et al.*, (1972) with little modifications. Each organism suspended in normal saline solution and a transmittance (T) of 75-77% at 530 nm was prepared. Plant extracts were dissolved in DMSO at a concentration of 100 mg/ml. Each plant extract was mixed with the melted media to make a final concentration of 5 mg/ml in every plate. The organisms were applied on the media as their inoculum (1 µl) by Cathra multipoint applicator. The plates were incubated at 35^0C for 24-96 h. The test was done in duplicates. The positive antifungal result was read based on no growth compared to solvent control (expressed as "++"). In the case of any growth, which was less than half in colony diameter of the solvent control, the result was reported as partial inhibition (expressed as "+").

Broth dilution: To measure the MIC (Minimum Inhibitory Concentration) values, recommendations of NCCLS (1992) were mostly used. Extracts were dissolved in DMSO to make a concentration of 250 mg/ml. The extracts were then diluted in a two-fold manner in the medium (SD broth) in 96 microwell plates, leaving 100 µl in each well. The fungal organisms, taken from SDA plates, were suspended in normal saline to obtain T%=75-77% at 530 nm, which is equal to 10^6 CFU/ml. The fungal suspension was diluted 1000 times in the medium and 100 µl aliquots were added to each well. The 96 well plates were incubated at 35^0C for 24-96 h. The MIC was defined as the minimum

concentration of the agent that shows a full inhibition of the fungus, examined by the naked eye. All tests were repeated two times.

Cell Toxicity Test

In vitro KB cell cytotoxicity (TD_{50}, µg/ml) was determined for a few of the extracts that showed better antifungal activity. The MTT method was used according to the Hansen *et al.*, (1989) procedure. The values are the average of three separate experiments.

Results

Table 2-2 summarizes the results of the antifungal test by the agar dilution method. As depicted, the extracts of *Bunium persicum, Diplotaenia damavandica, Heracleum persicum, Sanguisorba minor and Zataria multiflora* exhibit activity against the majority of the fungi tested. Out of a total of 40 plant species tested, 26 (65%) showed activity against at least one of the tested fungal species. The relative antifungal activity of the extracts against the fungi was observed in the following sequence: *A. niger* PLM 1140, 50%; *C. albicans* ATCC 14053, 27.5%; *A. fumigatus* PLM 712, 22.5%; *C. krusei* ATCC 44507, 22.5%; *C. galbrata* ATCC 90030, 20%. Among the plant extracts tested, Compositae with 8 active plants (80%), was ranked higher than the average in comparison to all plants studied. The second largest family in this study, Umbelliferae, exhibited only 6 bioactive members (66.6%), almost equal to the total average of all plants tested. Among the plants collected from Canada, only one out of five (20%) showed activity, however 25 (71.4%) of the plants from Iran were active. Different selection factors contributed to the percentage of antifungal plant as follows: 1) 55.5%; 2) 66.6%; 3) 60%; 4) 78.6%; 5) 65.2%; 6) 85.7%; 7) 58,3%; 8) 60%. On the basis of the above statistics, the most influential factor is 6 and the least is 1.

Some of the plant extracts used in this study, showed stimulatory effect on the growth of the fungi (Table 2-3). A total number of 19 plants (47.5%) showed growth stimulatory property on at least one of the tested fungi. In this respect, *Achillea millefolium, Nepeta glomerulosa , Pentatropis microphylla, Turgenia latifolia,* and *Zygophyllum Fabago* exhibited the most frequent stimulatory activity.

Table 2-1. Botanical name and other information on the plants studied for antifungal activity.

Family	Botanical Name	Collection Site	Plant part[a]	Selection Factor	Reference
Aceraceae	*Acer velutinum* Boiss. var. *glabrasence*	Fuman, Iran	L+F	5B, 6	Harun, *et. al.*, (1985); Dix, (1974); Von reis, *et. al.*, (1982)
	Acer velutinum Boiss. var. *Velutinum* Murr.	Fuman, Iran	L+F	5B, 6	Harun, *et. al.*, (1985); Dix, (1974); Von reis, *et. al.*, (1982)
Asclepidiaceae	*Pentatropis microphylla* (Roxb) Wight & Arm.	Ab-esk, Haraz, Iran	AP+Fl	4	
Compositae	*Achillea santolina* L.	Karaj, Iran	AP	1, 2, 3, 5, 7	Amin, (1991); Abdul-Latif, *et. al.*, (1985); Karamati, *et. al.*, (1993); Von reis, *et. al.*, (1982); Etman, *et. al.*, (1987); Barel, *et. al.*, (1991); Jaenson, *et. al.*, (1991); Salehi, *et. al.*, (1993)
	Achillea millefolium L.	Edmonton, Canada	AP	1, 2, 3, 5, 7, 8	McCutcheon, *et. al.*, (1994); Salehi, *et. al.*, (1993); Von reis, *et. al.*, (1982); Barel, *et. al.*, (1991)
	Artemisia annua L.	Lahijan, Iran	AP	1, 2, 3, 5, 7, 8	Amin, (1991); Zheng, (1994); Phillipson, *et. al.*, (1991); McCutcheon, *et. al.*, (1994); Abdul-Latif, *et. al.*, (1985); Salehi, *et. al.*, (1993); Von reis, *et. al.*, (1982); Altschul, (1973); Zani, (1991); Johnson, (1978)
	Calendula sp.	Karaj, Iran	AP	2A, 5A, 7	Amin, (1991)
	Centaurea iberica Trevir. ex Spreng.	Fuman, Iran	AP	5, 6, 8	Sevil, *et. al.*, (1984); Abdul-Latif, *et. al.*, (1985); Altschul, (1973); Cordy, (1978)
	Chrysanthemum leucanthemum L.	Edmonton, Canada	AP	2, 5, 7	Amin, (1991); Altschul, (1973); Von reis, *et. al.*, (1982)
	Echinops cephalotes DC.	Samgh-Abad, Taleghan, Iran	AP	2A, 5A, 7	Amin, (1991)
	Hertia angustifolia DC.	Ardestan, Iran	AP	1, 4, 5A, 8	Salehi, *et. al.*, (1993); Steyn, (1937)
	Tanacetum parthenium (L.) Schultz Bip	Rineh, Haraz, Iran	AP+Fl	2, 3, 5	Amin, (1991); Von reis, *et. al.*, (1982); Barsby, *et. al.*, (1993); Abdul-Latif, *et. al.*, (1985)
	Xanthium spinosum L.	Tehran, Iran	AP	1, 3, 5, 8	Salehi, *et. al.*, (1993); Ginesta, *et. al.*, (1994); Abdul-Latif, *et. al.*, (1985); Witte, *et. al.*, (1990)
Cupressaceae	*Juniperus excelsa* M.B.	Samgh-Abad, Taleghan, Iran	AP	2, 5B	Amin, (1991); McCutcheon, *et. al.*, (1994)
Datiscaceae	*Datisca cannabina* L.	Darband, Iran	AP	2B, 4, 8	Von reis, *et. al.*, (1982); Galey, *et. al.*, (1991)
Guttiferae	*Hypericum hyssopifolium* Vill.	Shahmirzad, Semnan, Iran	AP	1, 2, 5B	Salehi, *et. al.*, (1993); Amin, (1991); Altschul, (1973); McCutcheon, *et. al.*, (1994)

	Hypericum scabrum L.	Kandavan, Iran	AP	1, 2, 5B	Salehi, *et. al.*, (1993); Amin, (1991); Altschul (1973); McCutcheon, *et. al.*, (1994)
Labiatae	*Nepeta glomerulosa* Boiss	Namrood, Firouz-Kouh, Iran	AP	1, 2	Amin, (1991); Salehi, *et. al.*, (1993); Von reis, *et. al.*, (1982)
	Nepeta racemosa Lam.	Shahmirzad, Semnan, Iran	AP+Fl	1, 2, 4	Amin, (1991); Salehi, *et. al.*, (1993); Von reis, *et. al.*, (1982)
	Zataria multiflora Boiss	Abadeh, Shiraz, Iran	AP	1, 2A, 4	Amin, (1991); Salehi, *et. al.*, (1993)
	Ziziphora tenuior L.	Tehran, Iran	AP	2A, 4, 5B, 6, 8	Amin, (1991); Kazymov, *et. al.*, (1989); Altschul, (1973)
Leguminosae	*Colutea persica* Boiss	Kandavan, Iran	AP+Fl	4, 5B, 8	Aguinagalde, *et. al.*, (1990)
	Medicago sativa L.	Edmonton, Canada	AP	2A, 7, 8	Hooper, *et. al.*, (1937); Von reis, *et. al.*, (1982)
	Melilotus officinalis (Lam) L.	Edmonton, Canada	AP	2A, 7	Amin, (1991)
Loranthaceae	*Viscum album* L.	Noushahr, Iran	AP	2B, 3	Von reis, *et. al.*, (1982); Jurin, (1993)
Onagraceae	*Epilobium angustifolium* L. spp. *circumvagum*	Edmonton, Canada	AP	2B, 7	Altschul, (1973)
Plantaginaceae	*Plantago psyllium* L.	Zibakenar, Iran	AP	1, 2, 5B, 7	Amin, (1991); Von reis, *et. al.*, (1982); McCutcheon, *et. al.*, (1994); Salehi, *et. al.*, (1993)
Polygonaceae	*Polygonum amphibium* L.	Hasht-par, Iran	AP	2, 3, 5B	Hooper, *et. al.*, (1937); Von reis, *et. al.*, (1982); Altschul, (1973)
	Rumex pulcher L.	Abaali, Haraz, Iran	AP	1, 2, 4, 8	Amin, (1991); Von reis, *et. al.*, (1982); Altschul, (1973); Panciera, *et. al.*, (1990); Salehi, *et. al.*, (1993)
Rosaceae	*Hulthemia persica* J.F.Gmel.	Karaj, Iran	AP	1, 4, 5B	McCutcheon, *et. al.*, (1994); Salehi, *et. al.*, (1993)
	Sanguisorba minor Scop.	Karaj, Iran	AP	1, 2B, 6	Altschul, (1973); Salehi, *et. al.*, (1993)
Solanaceae	*Hyoscyamus niger* L.	Kandavan, Iran	AP+F	2A, 3, 5B	Amin, (1991); Khan, *et. al.*, (1992); Urkin, *et. al.*, (1991)
Tiliaceae	*Tilia platyphyllos* Scop. spp. *Caucasia*	Noushahr, Iran	Fl+B	2	Amin, (1991); Altschul, (1973)
Umbelliferae	*Bunium persicum* (Boiss) B. Fedtsch	Kerman, Iran	AP	2, 4, 3, 6, 7	Amin, (1991); Von reis, *et. al.*, (1982); Altschul, (1973); Khaidarov, *et. al.*, (1991)
	Diplotaenia damavandica Mozaffarian, Hedge & Lamond	Sarbandan, Damavand, Iran	AP	4, 8	
	Ducrosia anetifolia Boiss.	Ziyaran, Taleghan, Iran	AP	1, 3, 4	Salehi, *et. al.*, (1993)
	Echinophora platyloba DC.	Zanjan, Iran	AP+Fl	4, 5B	Kivanc, *et. al.*, (1990)
	Heracleum persicum Desf.	Plour, Haraz, Iran	F	1, 2, 4, 5B, 6, 7	Amin, (1991); McCutcheon, *et. al.*, (1994); Salehi, *et. al.*, (1993); Von reis, *et. al.*, (1982); Van de Sluis, *et. al.*,

					(1981)
	Turgenia latifolia Hoffm.	Rineh, Haraz, Iran	AP	1, 4	Salehi, *et. al.*, (1993)
Zygophyllacea	*Zygophyllum Fabago* L.	Ab-esk, Haraz, Iran	AP	1	Salehi, *et. al.*, (1993)

[a] AP, aerial part; B, brackts; F, fruit; Fl, flower; L, leaves

Table 2-2. Antifungal activity of the plant extracts.

Botanical Names	1[a]	2	3	4	5	6	7	8	9	10	11	12
Acer velutinum Boiss. var. *glabrasence*	-	-	nt[b]	-	nt	-	nt	nt	-	nt	nt	-
Acer velutinum Boiss. var. *Velutinum* Murr.	+	+	nt	+	nt	+	nt	nt	+	nt	nt	+
Achillea millefolium L.	-	-	-	-	-	-	-	-	-	-	-	nt
Achillea santolina L.	-	+	-	-	-	-	-	-	-	-	-	nt
Artemisia annua L.	-	++	-	-	-	-	-	+	-	-	-	nt
Bunium persicum (Boiss) B. Fedtsch	-	++	++	++	-	+	++	++	++	++	++	nt
Calendula sp.	++	++	-	-	-	-	-	-	-	++	-	nt
Centaurea iberica Trevir. ex Spreng.	+	-	nt	-	nt	-	nt	nt	-	nt	nt	++
Chrysanthemum leucanthemum L.	+	++	+	+	+	+	++	-	+	-	+	nt
Colutea persica Boiss	++	-	-	-	-	-	-	-	-	-	-	nt
Datisca cannabina L.	-	++	-	-	++	-	++	-	-	-	-	nt
Diplotaenia damavandica Mozaffarian, Hedge & Lamond	+	++	++	++	++	++	++	++	++	++	++	nt
Ducrosia anetifolia Boiss.	-	-	-	-	-	-	-	-	-	-	-	nt
Echinophora platyloba DC.	-	-	-	-	-	-	-	-	-	-	-	nt
Echinops cephalotes DC.	-	+	-	-	-	-	-	-	-	-	-	nt
Epilobium angustifolium L. spp. *circumvagum*	-	-	-	-	-	-	-	-	-	-	-	nt
Heracleum persicum Desf.	++	++	++	++	++	++	++	++	++	++	++	nt
Hertia angustifolia DC.	+	+	nt	-	nt	-	nt	nt	-	nt	nt	-
Hulthemia persica J.F.Gmel.	-	-	-	-	-	+	-	-	+	+	-	nt
Hyoscyamus niger L.	-	-	-	-	-	-	-	-	-	-	-	nt
Hypericum hyssopifolium Vill.	-	-	-	-	-	-	-	-	-	-	-	nt
Hypericum scabrum L.	-	-	-	-	-	-	-	-	-	-	-	nt
Juniperus excelsa M.B.	-	+	-	-	++	-	-	-	+	-	-	nt
Medicago sativa L.	-	-	-	-	-	-	-	-	-	-	-	nt
Melilotus officinalis (Lam) L.	-	-	-	-	-	-	-	-	-	-	-	nt
Nepeta glomerulosa Boiss	-	+	-	-	-	-	-	-	-	-	-	nt
Nepeta racemosa Lam.	-	+	-	-	-	-	-	-	-	-	-	nt
Pentatropis microphylla (Roxb) Wight & Arm.	-	++	-	++	-	-	++	-	-	-	-	nt

Plantago psyllium L.	-	-	nt	-	nt	-	nt	nt	-	nt	nt	-
Polygonum amphibium L.	-	-	-	++	-	-	-	-	-	-	-	nt
Rumex pulcher L.	-	-	-	-	-	-	-	-	-	-	-	nt
Sanguisorba minor Scop.	-	-	-	++	-	++	++	-	++	++	++	nt
Tanacetum parthenium (L.) Schultz Bip	-	++	-	++	-	-	-	-	-	+	++	nt
Tilia platyphyllos Scop. spp. *Caucasia*	-	++	-	-	-	-	-	-	-	-	-	nt
Turgenia latifolia Hoffm.	-	+	-	-	-	-	-	-	-	-	-	nt
Viscum album L.	++	++	-	++	-	-	-	-	-	-	-	nt
Xanthium spinosum L.	-	-	-	-	-	-	-	-	-	-	-	nt
Zataria multiflora Boiss	-	-	++	++	++	++	++	++	++	++	++	nt
Ziziphora tenuior L.	-	++	-	-	-	-	-	-	-	-	-	nt
Zygophyllum Fabago L.	-	-	-	-	-	-	-	-	-	-	-	nt
Fluconazole[c]	++	++	++	+	++	++	+	nt	+	nt	+	++
Amphotericin B[d]	+	++	++	++	+	++	nt	nt	++	nt	++	++
DMSO	-	-	-	-	-	-	-	-	-	-	-	+/-[e]

[a] 1. *Aspergillus fumigatus* PLM 712, 2. *Aspergillus niger* PLM 1140, 3. *Candida albicans* 200/175, 4. *Candida albicans* ATCC 14053, 5. *Candida albicans* Y01-09, 6. *Candida glabrata* ATCC 90030, 7. *Candida guilliermondi* IF00838, 8. *Candida kefyr* ATCC 38296, 9. *Candida krusei* ATCC 44507, 10. *Candida pseudotropicalis* Y06-01, 11. *Candida tropicalis* ATCC 13803, 12. *Cryptococcus neoformans* KF-33. [b] nt, not tested. [c] The final concentration was 50(µg/ml) in each plate. [d] The final concentration was 10 (µg/ml) in each plate. [e] In several cases, the DMSO control did not show growth.

Table 2-3. Growth stimulatory activity of the plant extracts[a].

Botanical Names	1[b]	2	3	4	5	6	7	8	9	10	11	12
Acer velutinum Boiss. var. *glabrasence*	-	-	-	-	-	-	-	-	-	-	-	-
Acer velutinum Boiss. var. *Velutinum* Murr.	-	-	-	-	-	-	-	-	-	-	-	-
Achillea millefolium L.	+	+	-	-	-	-	+	-	-	-	-	-
Achillea santolina L.	-	-	-	-	-	-	-	-	+	-	-	-
Artemisia annua L.	-	-	-	-	-	-	-	-	-	-	-	-
Bunium persicum (Boiss) B. Fedtsch	-	-	-	-	-	-	-	-	-	-	-	-
Calendula sp.	-	-	-	-	-	-	-	-	-	-	-	-
Centaurea iberica Trevir. ex Spreng.	-	-	-	-	-	+	-	-	-	-	-	-
Chrysanthemum leucanthemum L.	-	-	-	-	-	-	-	-	-	+	-	-
Colutea persica Boiss	-	-	-	-	-	+	-	+	-	-	-	-
Datisca cannabina L.	-	-	-	-	-	-	-	-	-	-	-	-
Diplotaenia damavandica Mozaffarian, Hedge & Lamond	-	-	-	-	-	-	-	-	-	-	-	-

Ducrosia anetifolia Boiss.	+	-	-	-	-	-	-	-	-	-	-	-
Echinophora platyloba DC.	-	-	-	-	-	-	-	+	-	+	-	-
Echinops cephalotes DC.	+	-	-	-	-	+	-	-	-	-	-	-
Epilobium angustifolium L. spp. *circumvagum*	+	+	-	-	-	-	-	-	-	-	-	-
Heracleum persicum Desf.	-	-	-	-	-	-	-	-	-	-	-	-
Hertia angustifolia DC.	-	-	-	-	-	-	-	-	-	-	-	-
Hulthemia persica J.F.Gmel.	-	-	-	-	-	-	-	-	-	-	-	-
Hyoscyamus niger L.	-	-	-	-	-	+	-	+	-	-	-	-
Hypericum hyssopifolium Vill.	-	-	-	-	-	-	-	-	-	-	-	-
Hypericum scabrum L.	-	-	-	-	-	-	-	-	-	-	-	-
Juniperus excelsa M.B.	-	-	-	-	-	-	-	-	-	-	-	-
Medicago sativa L.	-	-	-	-	-	-	-	-	-	+	-	-
Melilotus officinalis (Lam) L.	-	-	-	-	-	-	-	-	-	-	+	-
Nepeta glomerulosa Boiss	-	-	-	-	-	+	-	-	+	-	-	-
Nepeta racemosa Lam.	-	-	-	-	-	-	-	-	-	-	-	-
Pentatropis microphylla (Roxb) Wight & Arm.	-	-	-	-	-	+	-	+	+	-	-	-
Plantago psyllium L.	-	-	-	-	-	-	-	-	-	-	-	-
Polygonum amphibium L.	-	-	-	-	-	-	-	-	-	-	-	-
Rumex pulcher L.	-	-	-	-	-	+	-	+	-	-	-	-
Sanguisorba minor Scop.	-	-	-	-	-	-	-	-	-	-	-	-
Tanacetum parthenium (L.) Schultz Bip	-	-	-	-	-	-	-	-	-	-	-	-
Tilia platyphyllos Scop. spp. *Caucasia*	-	-	-	-	-	-	+	-	-	-	-	-
Turgenia latifolia Hoffm.	-	-	-	-	-	+	-	+	-	+	-	-
Viscum album L.	-	-	-	-	-	-	-	-	-	-	-	-
Xanthium spinosum L.	-	-	-	-	-	+	-	+	-	-	-	-
Zataria multiflora Boiss	-	-	-	-	-	-	-	-	-	-	-	-
Ziziphora tenuior L.	-	-	-	-	-	-	-	-	-	-	-	-
Zygophyllum Fabago L.	-	-	-	-	-	+	-	+	-	+	-	-

[a] Growth stimulation compared with the solvent (DMSO) control plates. Any growth of about more than twice in the diameter of the fungal colony is reported here. [b] 1. *Aspergillus fumigatus* PLM 712, 2. *Aspergillus niger* PLM 1140, 3. *Candida albicans* 200/175, 4. *Candida albicans* ATCC 14053, 5. *Candida albicans* Y01-09, 6. *Candida glabrata* ATCC 90030, 7. *Candida guilliermondi* IF00838, 8. *Candida kefyr* ATCC 38296, 9. *Candida krusei* ATCC 44507, 10. *Candida pseudotropicalis* Y06-01, 11. *Candida tropicalis* ATCC 13803, 12. *Cryptococcus neoformans* KI-33.

Table 2-4. MIC values (mg/ml) of several plant extracts in the microwell broth dilution assay.

Botanical name	1[a]	2	3
Achillea santolina L.	6.25	-	3.125
Bunium persicum (Boiss) B. Fedtsch	3.125	1.56	≤0.195
Centaurea iberica Trevir. Ex Spreng.	-[c]	nt[e]	1.56
Diplotaenia damavandica Mozaffarian, Hedge & Lamond	3.125	1.56	≤0.195
Ducrosia anetifolia Boiss.	-	-	3.125
Echinops cephalotes DC.	-	-	3.125
Hertia angustifolia DC.	-	Nt	-
Hulthemia persica J.F.Gmel.	1.56	0.78	≤0.195
Hypericum scabrum L.	3.125	1.56	3.125
Juniperus excelsa M.B.	3.125	1.56	3.125
Nepeta racemosa Lam.	-	Nt	-
Plantago psyllium L.	-	Nt	-
Sanguisorba minor Scop.	≤0.195	≤0.195	≤0.195
Zataria multiflora Boiss.	0.78	0.78	0.39
DMSO	6.25[d]	6.25	3.125
Fluconazole[b]	2.5	2.5	≤0.78

[a] 1. *Candida albicans* ATCC 14053, 2. *Candida krusei* ATCC 44507, 3. *Cryptococcus neoformans* KF-33.
[b] The highest concentration was 100 µg/ml.
[c] The sign "-" means that the agent did not show any activity even at the highest concentration tested, which was 25 mg/ml for the extracts.
[d] The value is expressed in v/v%.
[e] not tested

Table 2-5. *In vitro* cytotoxicity of some antifungal plant extracts against KB cells.

Plant name	TD_{50} (µg/ml)[a]
Bunium persicum (Boiss) B. Fedtsch	474.64
Diplotaenia damavandica Mozaffarian, Hedge & Lamond	419.05
Sanguisorba minor Scop.	304.24
Zataria multiflora Boiss.	284.62
Adriamycin[b]	0.03

[a] Cytotoxic concentration in 50% of the cells.
[b] Reference drug.

Several active plant extracts were tested for their MIC and cell toxicity. The MIC values are shown in Table 2-4. Among the plant extracts tested for their MIC, *Bunium persicum*, *Diplotaenia damavandica*, *Hulthemia persica*, *Sanguisorba minor*, *Zataria multiflora* showed the lowest values. *In vitro* KB cell toxicity test (Table 2-5) showed high TD_{50} values for the tested plant extracts. Two plants, namely *D. damavandica* and *B. persicum* are especially low in toxicity.

Discussion

According to findings from the National Nosocomial Infection Surveillance System (NNIS), 61% of reported nosocomial fungal infections were due to *Candida albicans* (Walsh 1992). Other *Candida* spp., and *Aspergillus* spp. accounted for 18.9% and 1.3% of the cases. Therefore, in this study, different strains of these fungi were employed.

The results reported here are affected by factors such as the population of the tested plant, type of extraction, and the antifungal test method. Therefore, one may expect discrepancy in the results among the reported activity for one species of plant. The presence of activity of the extracts used in the preliminary tests may well depend on the concentration. For example, concentrations of the extract from as low as 100 µg (Rahalison, *et al.*, 1993) to as high as 20 mg/ml (Hufford, *et al.*, 1975) has been used in each test. Another factor that should be considered is the application of light in antifungal testing. The activity of some plant constituents is mediated by light (Van de Sluis, *et al.*, 1981). Among these, *Hypericum* species and other genera belonging to Umbelliferae and Compositae can be mentioned.

To find a better method of acquiring antifungal plants, a non-random method was chosen in this study. Each plant was selected based on one or a combination of eight factors. Most of the antifungal plants representing factor 6 followed by factors 4 and 2 in their selection. The least contributing factor was number 1. There might not be a clear explanation for this result. However, since factor 1 is the contribution of activity against

dermatophytes, it may be conceivable to expect a low number of activities against systemic fungi like *Candida*.

The growth stimulatory activity of the plant extracts was clearly observed for many of the plants (Table 2-3). This effect is an already known phenomenon (Griffin, 1994). For example, in addition to nutrients like carbohydrates, other compounds and agents usually in low quantities can have growth increasing activity. Sterols, aliphatic aldehydes, organic acids, and flavonoids are among the groups that can be growth stimulants. The importance of these compounds is that they may antagonize the antifungal activity of other ingredients present in the extract. Depending on the concentration and other factors, net observed activity can be growth inhibition, stimulation or no activity.

The MIC values provide a better means of comparing the active extracts, however, since this test is a sensitive method (Sheehan, *et al.*, 1993; Chand, *et al.*, 1994). Several plants have shown antifungal activity that was not observed using the agar dilution technique. The overall agreement between these two methods is considerable. Precipitation of the extracts in higher concentrations that hinders proper reading of the MIC is a disadvantage for this method.

To examine the selectivity of the effective antifungal plants, *in vitro* KB cell cytotoxicity was performed. Although, this test is done on a nasopharyngeal carcinoma cell line (KB), it can well be indicative of how toxic an agent might be in an *in vivo* animal test or in human. In this test, a TD_{50} value above 100 (μg/ml) is usually interpreted to be in the non-toxic range. Therefore, the extracts from *D. damavandica* and *B. persicum* were especially interesting and considered for more detailed study.

As the ethnobotanical analysis has demonstrated, the selection of plants based on traditional usage appears to increase the probability of acquiring plants with antifungal activity (McCutcheon, *et al.*, 1994). In our study, we have expanded this view to other guiding factors like being a food or spice, chemotaxonomy, rarity, and ecology. Therefore, not only the possibility of acquiring an antifungal plant is higher, but also by

considering several factors together, one may expect a good chance of finding a more promising extract.

In the medical doctrine established and practiced by Avicenna (980-1037 A.D.), diseases as well as medicines have specific temperaments (Shah, 1966; Sharafkandi, 1991). In this way, the four temperaments of Dry, Moist, Hot, and Cold or any mixture of them is assigned for each specific medication. These temperaments are similar to Chinese cosmic elements, fire, water, earth, metal, and wood which are nowadays believed to have originated from the Iranian version, fire, water, earth, metal, and plant (Mahdihassan, 1989). The degree of each temperament is expressed by numbers, e.g., H_1D_3 represents a degree of one for heat and three for dryness; the higher the number the stronger the effect. The plants from Avicenna's practice that were included in our survey and their temperament are shown in Table 2-6. As can be seen, most of the plants with Hot and Dry temperament show antifungal activity, and almost all the active plants are in the Dry group. A clear relationship can not be seen between the spectrum of activity and the degree of temperaments. Although the number of plants studied is limited, this result is a good idea for more research on this respect. As our program of finding new leads from natural sources mentioned in the traditional medicine of Iran continues, we currently tend to test more plants based on the temperament classification.

Table 2-6. Antifungal activity and temperament of the plants studied, as mentioned by Avicenna.

Plant name	Temperament	Antifungal activty	Number of sensitive fungal strains
Artemisia	H_1D_3, H_3D_3, H_1D_2 [a]	+	2
Bunium	H_2D_3	+	9
Hyoscyamus	C_3D_3	-	0
Juniperus	H_3D_3	+	3
Melilotus	H_1D_1	-	0
Nepeta	H_3D_3	+	1
Plantago	C_2D_2, C_2M_2[a]	-	0
Rumex	C_2D_1	-	0
Zataria	H_3D_3	+	9

[a] Different species

The high percentage of active plants found in this study might be a direct result of non-random selection strategy. In addition to identification of new sources for antifungal activity, the spectrum of activity and selectivity of this property are important for future studies for finding efficient antifungal lead structures. Traditional medicine as a whole, and that of Iran particularly, demonstrate excellent potential in this area of research.

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Chapter 3

Phytochemical and Biological Studies of *Diplotaenia Damavandica*

Introduction

In the process of active research on natural sources, the isolation of bioactive compounds is usually considered to be an important step. In a normal drug development course, after primary screening of a large group of extracts, secondary screening and bioassay identification of active fractions and compounds are performed. Secondary screening, which is an expanded version of primary bioactivity assay, is in fact guiding the researcher to have a better idea of the profile of activity, i.e., potency, selectivity, efficacy, toxicity and so on. As far as primary screening is concerned, the detailed evaluation was performed as represented in chapter 2. Several preliminary steps in the evaluation of plant extracts for their potency, selectivity, and toxicity were taken. The purpose of this research is to carry out further studies on the profile of activity and isolation of active ingredients of one of the successful plants that was selected based on the previous results.

Diplotaenia damavandica Mozaffarian, Hedge & Lamond (Umbelliferae) is a perennial herb growing wild almost exclusively in Mount Damavand in the North Central part of Iran (Reichinger, 1987). This plant showed interesting antifungal activity in our study (Chapt. 2). *D. damavanica* is also a very rare plant with a narrow niche, covering the high altitude of a volcanic mount in Iran (Mount Damavand). Several factors influenced selection of this plant. Due to the apparent skin reaction activity of this plant on the grazing animals like sheep, observed in the field, rarity of the plant, and its *in vitro* bioactivity, it was decided to perform further study on its constituents and antifungal effects.

Materials and Methods

Plant material

The plant was collected in May 1997 on Mount Damavand (Fig. 3-1) (Province of Tehran), Iran and air-dried. Taxonomic identification was established by Dr. Gh. Amin, Curator of the Herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Iran, where a voucher specimen is deposited.

Figure 3-1. Mount Damavand area (a volcanic mount), which is the growth habitat of *D. damavandica* in the North-Central part of Iran. It should be noted that this plant is dominant in the area.

Botany of *D. damavandica*: This plant with the common name "Kozal", is a perennial herb, approximately 120 cm tall (Fig. 3-2). It has pinnate leaves, ovate-oblong shape, about 60 cm long by 40 cm wide (Fig. 3-3). The umbels are subsessile terminal (Fig. 3-4). It is native to Iran (Persia) and grows wild on Mount Damavand, (Tar Lake and Garrubar Valley) at the altitude of 2450-3000 m. *D. damavandica* shares with other species of *Diplotaenia* the verticillate branching, but clearly differs in the shorter, broader leaf segments and apparently in having special oil tubes in the carpel (Reichinger, 1987).

Figure 3-2. A group of *D. damavandica* or Kozal plants in full bloom.

Figure 3-3. A close-up picture of compound leaves in *D. damavandica*.

Figure 3-4. Inflorescence of *D. damavandica* with typical umbrella arrangement and white petals.

Extraction, isolation and identification of compounds

The above ground parts (200 g) were extracted with methanol. Evaporation of the extract left residue **1** (31 g). About 5.1 g of **1** was subjected to flash chromatography on silica gel with ether to obtain 2.6 g of residue **2**. Ether was selected according to the results obtained in TLC agar overlay test, since the inhibition zone in this method had an increasing Rf on silica gel plates as the amount of ether in dual mixture mobile phase ($CHCl_3$:ether) was increased. The fractionation was guided by *in vitro* antifungal assessment. Silica gel column chromatography of **2** with a gradient of 2% to 100% methanol in chloroform yielded fractions **3-9**. Fraction **7** (360 mg) was rechromatographed to achieve **10-15.** Fraction **12** (217 mg) was chromatographed, using 80% to 10% light petroleum spirit in chloroform yielding **16-19**. To obtain fractions **21-24, 16** (170 mg) was fractionated by MPLC (4 × 33 cm) on silica gel (20-45 μm) with petroleum spirit 16% to 10% in chloroform, 5 ml/min. Fraction **21** (95 mg) was

rechromatographed by MPLC to yield **25** (angelicin) 11mg, **26** (45 mg), **28** (12 mg), **29** (libanorin) 18mg. Fraction **26** was further purified by HPLC (Shimadzu, CC 610 Liquid Chromatograph; column Zorbax 9.4 mm i.d.×25 cm; detector, Shimadzu SPD-6AV UV-Vis, at 259 nm; mobile phase, 90% hexane in ethyl acetate, with a flow rate of 4 ml/ min); psoralen (1 mg) was eluted at 35min. **30** (auraptene) 3 mg was obtained by purifying **28** using preparative TLC and 5% methanol in chloroform.

Angelicin White crystalline compound, mp 140-143 ^{0}C (uncorr.). HI-MS m/z (%): 186.03194 (100.0) M^+ ($C_{11}H_6O_3$, cald. 186.03169), 158.04 (89.9), 149.02 (26.6), 130.04 (13.5), 102.05 (23.5). UV λ^{MeOH} nm (log ε): 251 (4.4), 301 (4.0). IR ν ($CHCl_3$ cast) cm $^{-1}$: 1727.1, 1651.2, 1617.2, 830.7, 747.7. ^{1}H-NMR ($CDCl_3$) δ: 7.83 (1H, d, J = 9.6, H-4), 7.71 (1H, d, J = 2.1, H-2'), 7.45 (1H, dd, J = 8.7, 1.1, H-6), 7.39 (1H, d, J = 8.5, H-5), 7.15 (1H, dd, J = 2.1, 1.4, H-3'), 6.41 (1H, d, J = 9.6, H-3). ^{13}C-NMR (see Table 3-1).

Libanorin Colorless greasy solid, mp 78-79° C (uncorr.), $[\alpha]^{24}D$ +193.34^0 (*c*= 0.72, $CHCl_3$). HI-MS m/z (%): 328.13137 (9.7) M^+ ($C_{19}H_{20}O_5$, cald. 328.13107), 246.3 (1), 228.1 (41.3), 213.1 (100), 187.0 (11.9), 83.1 (54.9). UV λ^{MeOH} nm (log ε): 237 (3.6), 329 (4.2). IR ν ($CHCl_3$ cast) cm $^{-1}$: 1735.8, 1718.7, 1617.4, 1455.8, 1385.6, 1262.9, 1068.8, 832.5. ^{1}H-NMR ($CDCl_3$) δ: 7.64 (1H, d, J = 9.5, H-4), 7.27 (1H, d, J = 8.1, H-6), 6.75 (1H, d, J = 8.2, H-5), 6.22 (1H, d, J = 9.5, H-3), 5.57 (1H, m, H-8'), 5.21 (1H, dd, J = 8.0, 8.2, H-2'), 3.36 (2H, m, H-3'), 2.1 (3H, d, J = 0.8, H-10'), 1.86 (3H, d, J = 0.8, H-11'), 1.61 (3H, s, H-5'), 1.53 (3H, s, H-6'). ^{13}C-NMR (see Table 3-1).

Psoralen white amorphous powder, mp 165-167^{0}C (uncorr.). HI-MS m/z (%): 186.03158 (100) (cald. $C_{11}H_6O_3$, 186.03169), 158.0 (89.1), 130.0 (24.2), 102.0 (43.4), 77.1 (21.3). UV λ^{MeOH} nm (log ε): 248 (4.42), 295 (4.00), 332 (3.38) sh. IR ν ($CHCl_3$ cast) cm $^{-1}$: 1720.5, 1663.2, 838.8, 754.2. ^{1}H-NMR ($CDCl_3$): δ 7.81 (1H, d, *J* = 9.6 Hz, H-4), 7.71 (1H, d, *J* = 2.5 Hz, H-2'), 7.69 (1H, s, H-5), 7.49 (1H, s, H-8), 6.84 (1H, d, *J* = 2.5 Hz, H-3'), 6.39 (1H, d, *J* = 9.6 Hz, H-3). ^{13}C-NMR (see Table 3-1).

Auraptene [(+)-Meranzin] Pale yellow amorphous powder, mp 98-99^0 C (uncorr.), $[\alpha]^{25}D$ +1.3^0 (*c*= 0.25, $CHCl_3$). HI-MS m/z (%): 260.10440 (6.7) M^+ ($C_{15}H_{16}O_4$, cald. 260.10486), 245.1 (22), 220.1 (33.5), 189.0 (24.3), 177.1 (100.0), 131.0 (22.5). UV λ^{MeOH} nm (log ε): 257 (3.6), 322 (4.5). IR ν ($CHCl_3$ cast) cm^{-1}: 1717, 1588, 1454. ^{1}H-NMR ($CDCl_3$): δ 7.65 (1H, d, *J* = 9.5 Hz, H-4), 7.37 (1H, d, *J* = 8.2 Hz, H-6), 6.89 (1H, d, *J* = 8.3 Hz, H-5), 6.27 (1H, d, *J* = 9.5 Hz, H-3), 3.96 (3H, s, OMe), 3.67 (1H, dd, *J* = 10.7, 2.1 Hz, H-3'), 3.14 (1H, dd, *J* = 2.1, 13.7 Hz, H-2'a), 3.00 (1H, dd, *J* = 10.7, 13.7 Hz, H-2'b), 1.36 (3H, s, H-5'/6'), 1.35 (3H, s, H-6'/5'). ^{13}C-NMR (see Table 3-1).

Biological activity

Microwell broth dilution method: The antifungal activity was measured based on the recommendations of NCCLS (National Committee for Clinical Laboratory Standards, 1992). The fractions or the compounds were dissolved in acetone and diluted in a two-fold manner in SD broth in 96 microwell plates. The MIC was the minimum concentration of the agent that shows a full inhibition of the fungal growth in the well, examined by the naked eye.

TLC agar overlay method: This method was adopted from the previous reports (Homans, *et al.*, 1970; Peterson, *et al.*, 1969; Hamburger, *et al.*, 1987; Rahalison, *et al.*, 1991). 1-100 µl of a 1mg/ml solution of plant extract in MeOH was applied on silica gel TLC plates and developed with 10% and 30% ether in $CHCl_3$ and 100% ether. The molten SDA medium maintained in a water bath at 45^{0}C. The fungal organism (here *C. albicans* ATCC 14053 and *Cladosporium cucumerinum* ATCC 64202) were added to the medium separately to make two suspensions, each containing approximately 10^5 cells/ml (T=75-77% measured by spectrophotometer at 530 nm is roughly equal to 10^6 cells/ml). Approximately 5 ml of the inoculum was rapidly distributed over the TLC plate with a sterile micropipette. After solidification of the medium, TLC plates were incubated overnight at 30^{0}C in sterile polyethylene dishes lined with sterile moist chromatography paper. The bioautograms were sprayed with or dipped into an aqueous solution (2.5 mg/ml) of thiazolyl blue (methylthiazolyltetrazolium chloride; MTT) (Sigma), and incubated for 3 h at 30^{0}C. Clear inhibition zones were observed. In the case of

Cladosporium cucumerinum, there is no need for MTT, since the fungal lawn has a gray color makeing it possible to see the inhibition zones. The extract *of D. damavandica* tested in this method showed a clear zone of inhibition with increasing Rf as the amount of ether was increased in the mobile phase.

Cytotoxicity test: *In vitro* KB cell toxicity was determined for a few of the fractions and pure compounds which showed better antifungal activity. The MTT method was used for the cell toxicity test, which involves conversion of MTT to blue colored formazan derivative by the active cells, according to the Hansen *et al.* (1989) procedure. The values are average of three separate experiments and expressed as TD_{50}, µg/ml.

Table 3-1. ^{13}C NMR spectral data for angelicin, libanorin, psoralen and auraptene (in CD_3OD, 100 MHz).

Carbon	Angelicin	Libanorin	Psoralen	Auraptene
2	160.8	164.0	161.1	161.0
3	114.2	116.9	114.8	113.4
4	144.4	143.9	143.9	143.8
5	123.8	128.8	119.8	127.1
6	108.8	106.7	124.9	107.4
7	157.4	161.0	156.6	160.1
8	116.9	113.0	99.9	114.8
9	148.5	156.5	152.2	156.7
10	113.5	113.6	115.5	113.0
2'	145.9	89.1	146.9	25.7
3'	104.1	27.6	106.4	78.4
4'		81.8		82.3
5'		21.1[a]		23.9[a]
6'		20.0[a]		26.2[a]
7'		165.8		
8'		112.2		
9'		151.5		
10'		27.3[a]		
11'		22.3[a]		
OMe				56.33

[a] Assignment might be interchangeable (within each column)

Results

Identification of the constituents was established by spectroscopy and by comparison with literature and authentic samples (Figures 3-6 and 3-7); data were in agreement with the reported values for angelicin (Seshadri, et.al., 1967; Steck *et al.*, 1972; Batterham, *et al.*, 1964; Bose, *et al.*, 1979; TRC Spectral Data-Mass, 1992), libanorin (Ermatov, *et al.*, 1969; Perel'son, *et al.*, 1971; Bohlmann, *et al.*, 1969; Bohlmann, *et al.*, 1968), psoralen (Steck, *et al.*, 1972; Kutney, *et al.*, 1972; Elgamal, *et al.*, 1979), and auraptene (meranzin) (Grundon, *et al.*, 1975; Raj, *et al.*, 1976) (Fig. 3-5). The isolated coumarins from *D. damavandica*, are reported from this source for the first time.

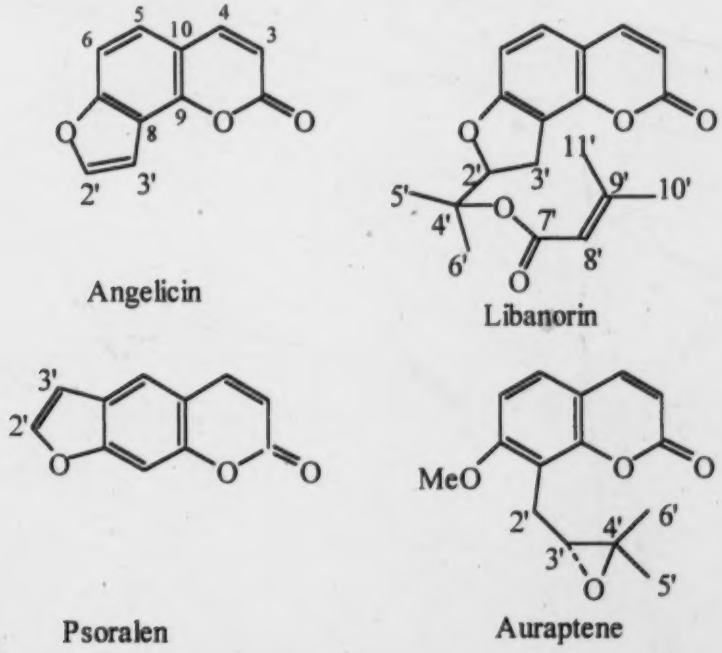
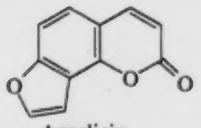
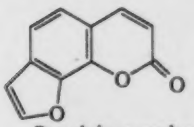


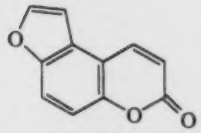
Figure 3-5. Structure of the coumarin compounds isolated from *D. damavandica*.



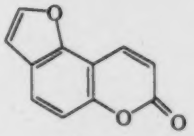
Angelicin



Pseudoisopsoralen



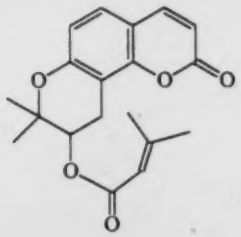
Isopseudopsoralen



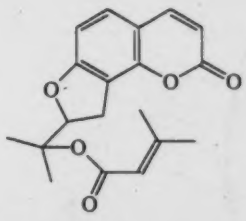
Allopsoralen

Hydrogen ($CDCl_3$)	Angelicin	Pseudoisopsoralen	Isopseudopsoralen	Allopsoralen
H-3	6.42 (J = 9.8)	6.43 (J = 9.6)	6.54 (J = 9.6)	6.54 (J = 9.5)
H-4	7.83 (J = 9.8)	7.83 (J = 9.6)	8.03 (J = 9.6)	8.22 (J = 9.5)
H-5	7.40 (J = 8.5)	7.48 (J = 8.2)		
H-6	7.46 (J = 8.5, 0.9)	7.33 (J = 8.2)		
H-7			7.31 (J = 8.9)	7.27 (J = 8.5)
H-8			7.68 (J = 8.9)	7.73 (J = 8.5)
H-2'	7.72 (J = 2.1)	7.82 (J = 2.1)	7.82 (J = 2.1)	7.74 (J = 2.3)
H-3'	7.16 (J = 2.1, 0.9)	6.90 (J = 2.1)	7.04 (J = 2.1)	6.87 (J = 2.3)

Figure 3-6. Structure and ^{1}H NMR values for angelicin and its different isomers.



Nuttallin (Buchtormin)



Libanorin

Compound	Nuttalin[1]	Libanorin[2]	Found
Mp ^{0}C	58-62	78-79	78-79
$[\alpha]_D$	75	213	193
MS	328, 313, 185	328, 246, 187	328, 246, 187

[1] Bohlman, *et al.*, 1968; Lee, *et al.*, 1968; [2] Bohlman, *et al.*, 1969; Ermatov, *et al.*, 1969

Figure 3-7. Structure and physical/spectral data for libanorin and its isomer, nuttallin.

As indicated in Table 3-2, many antifungal fractions were found in the *D. damavandica* extract. Bioassay guided isolation of the main fractions led to the purification of angelicin as an antifungal coumarin. The other coumarin derivative, libanorin, was not active in our test system. Auraptene showed moderate activity against *Cryptococcus*. Psoralen was not tested due to the low isolation yield.

Table 3-2. Antifungal activity of active fractions of *D. damavandica* in terms of MIC values (μg/ml). Medium, SD broth; temperature 30^0C; inoculum 10^3 CFU/ml; incubation 24-48 hours.

Fraction/ Compound	*Candida albicans* ATCC 14053	*Cryptococcus neoformans* KF-33	*Cladosporium cucumerinum* ATCC 64202
1	3128	195	Nt
2	156.25	16.25	Nt
5	-	312.5	312.5
6	312.5	312.5	312.5
7	312.5	312.5	312.5
12	Nt[1]	75.3	156.3
13	Nt	312.5	-
16	100	125	125
21	Nt	78.1	156.3
Angelicin	125	62.5	62.5
Libanorin	-[2]	-	-
26	62.5	62.5	62.5
28	250	-	125
Auraptene	-	250	Nt
AmB	0.1	0.39	0.185

[1] Nt, not tested; [2] "–", no activity

Human cell toxicity test showed that neither angelicin nor the total fractions **1** and **2** were cytotoxic (Table 3-3). As can be seen, the level of cytotoxicity found for angelicin as well as fractions **1** and **2**, is much less than adriamycin as the standard cytotoxic agent.

Table 3-3. In vitro cytotoxicity of several fractions and pure compounds obtained from *D. damavandica* against the KB cell line. The values are TD_{50} (μg/ml).

Fraction/Compound	TD_{50}
1	419.05
2	504.08
25 (angelicin)	90.63
Adriamycin	0.04

Discussion

Although there are previous reports on the light induced antifungal activity of coumarins against plant pathogenic fungi (Martin, *et al.*, 1966; Weimarck, *et al.*, 1980; Jüttermann, *et al.*, 1985; Van der Sluis, *et al.*, 1981; Zhang, *et al.*, 1981), here we reported these activities against human opportunistic fungi, and in the absence of UV light. In the case of psoralen, which has not been tested here, there are reports on the antifungal activity in the presence (Mikkelson, *et al.*, 1961) and absence of UV light (Chakraborty, *et al.*, 1957). No report on the antifungal testing of libanorin and auraptene has been found.

Recently emphasis has been placed on the evaluation of natural products as effective antifungals that may be devoid of adverse side-effects in human therapy. In the cytotoxicity tests, the main antifungal fraction and angelicin, which showed relatively strong activity, had no human cell toxicity. In contrast to angelicin, auraptene has shown cell toxicity against several cell lines (Satoh, *et al.*, 1996). Selective activity of angelicin against fungal cells made it more interesting as a target for further research and prompted us to synthesize and test several derivatives.

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Chapter 4

Phytochemical and Biological Studies of *Fraxinus Americana*

Introduction

The tree, *Fraxinus americana* L. belongs to the family Oleaceae and is native to the United States and Canada. Leaves and bark of different species have been used in the treatment of various ailments (Kruedener, *et al.*, 1995). There are traditional uses of the bark of *Fraxinus* (native name, "agimak"). In Appalachia, the chewed bark is applied on sores as a poultice; tea made from buds is useful for snakebite; seeds are believed to be an aphrodisiac and increase appetite and urine flow; the bark taken internally is supposed to increase perspiration and urine flow (Krochmal, *et al.*, 1984). Also *Fraxinus* sp. is used as a tonic by Chippewa Indians (Densmore, 1974).

There are many reports of the isolation of bioactive molecules from *Fraxinus* sp. (Jung, *et al.*, 1965; Nishibe, *et al.*, 1986, Nishibe, 1989). A lack of full knowledge as well as considerable doubt (Millspaugh, 1974) about the principal constituents of *F. americana* bark were the major driving forces to pursue the present research. In this report we discuss the isolation of several compounds from the bark of *F. americana*, which is to the best of our knowledge for the first time. Also the studied biological activities are not reported before. In addition, this research is a good starting point to find out any rational relationship between the major constituents and the traditional uses of this plant. Likewise, the antifungal and enzyme inhibitory activities were tested with the aim of clearing the potential usage of the isolated compounds and the plant itself in therapy.

Materials and Methods

Plant materials: Cultivated white ash (female) branches (2-3 years old) of *Fraxinus americana* L. were collected in Sept. 1995 in Edmonton, Canada. The voucher specimens

have been deposited in the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido.

Botany of F. americana L. (Oleaceae): *F. americana*, with the common name "white ash", has a wide distribution in the Eastern N. American region. It is a large tree, 24 m height, and 0.6 m diameter; leaves are opposite, pinnately compound, 20-30 cm long, with 5-9 leaflets (Fig. 4-1) (Little, 1995). Bark is dark gray, thick, with diamond-shaped furrows and forking ridges (Fig. 4-2). Flowers are 6 mm, purplish, without corolla, many in small clusters. The fruits are 2-2.5 cm long, brownish, with narrow wings, not extending down cylindrical body (Little, 1995).

Figure 4-1. The fruit-bearing twig of *F. americana* (white ash).

Figure 4-2. Pieces of bark of *F. americana* used for extraction in this study.

Isolation and Identification: *F. americana* bark powder (500 g) was extracted with boiling methanol at 40^0C, three times, with 1500 ml for one hour each. After filtration, about one third of the solvent was evaporated. Then 1200 ml water was added and the precipitate (2.5 g) was separated by filtration. The extraction was continued on the hydro-alcoholic solution by using ether (2000 ml), EtOAc (1700 ml), and *n*-butanol (1500 ml) successively. The yield of each of the fractions was as follows: ether (1.68 g), EtOAc (9.06 g), and n-butanol (47.14 g). A residue of 34.05 g remained in the aqueous phase.

The EtOAc fraction (1g) was chromatographed on Sephadex LH-20 with distilled water, continued to add increasing amount of EtOH, starting from fraction 16, to finally 100% EtOH; steps of 5% and 300 ml each were chosen, and for each fraction a volume of 100 ml was collected. Detection was done mostly by silica gel TLC and EtOAc:MeCOEt:C_6H_6:HCOOH (4:3:1:2) as the mobile phase.

Verbascoside (97 mg) was eluted with 10% EtOH in H_2O. Fractions 6-7 (400 mg) and 9-10 (155 mg) were re-chromatographed on Sephadex LH-20 washed with water to give 10-hydroxyligstroside (362 mg) and ligstroside (109 mg). Syringin (25 mg) was obtained directly from the n-BuOH fraction by crystallization and subsequent washing with ether, water and acetone.

HPLC: The methanolic solution of each fraction or pure compound (5 µl) was injected directly into the HPLC. The HPLC system included a Shimadzu liquid chromatograph LC-9A with a UV-visible detector, Shimadzu SPD-10A and a reversed phase Develosil ODS-5 column, 4.6×250 mm, kept at 35°C. Detection was done at 280 nm. In the HPLC of coumarins and phenolic compounds a mobile phase of acidic water and methanol is recommended (Nykolov, *et al.*, 1993). The column was eluted with isocratic solution MeOH:H_2O:HOAc (8:30:2) as the mobile phase. The flow rate was equal to 0.5 or 1 ml/min. Retention times (Rt, min) were as follows: **1,** 44.48 (0.5 ml/min), 23.78 (1 ml/min); **2,** 12.17 (0.5 ml/min), 5.57 (1 ml/min); **3,** 145.45 (1 ml/min), **4,** 86.52 (0.5 ml/min), 45.38 (1 ml/min).

X-ray crystallography:

Data collection: A colorless prismatic crystal of $C_{17}H_{24}O_9$ having approximate dimensions of 0.15 × 0.15 × 0.25 mm was mounted on a glass fiber. All measurements were made on a Rigaku RASA diffractometer with graphite monochromated Cu-Kα radiation and a 18 kW rotating anode generator. Cell constants and an orientation matrix for data collection, obtained from a least-square refinement using the setting angles of 25 carefully centered reflections in the range $50.62 < 2\theta < 56.8°$ corresponded to a primitive orthorhombic cell with dimensions:

a = 19.4933(7) Å, b = 20.0703(8) Å, c = 4.854(1) Å, V = 1899.1(5) Å^3

For Z = 4 and F.W. = 372.37, the calculated density is 1.30 g/cm^3. The systematic absences of:

h00: $h \neq 2n$, 0k0: $k \neq 2n$, 00l: $l \neq 2n$

The data were collected at a temperature of 20 ± 1°C using the ω-2θ scan technique to a maximum 2θ value of 120.1°. The ratio of peak counting time to background counting

time was 235 mm, and the computer controlled detector aperture was set to 9.0 × 13.0 mm (horizontal × vertical).

Data reduction: A total of 1719 reflections were collected. The intensities of three representative reflections were measured after every 150 reflections. No decay correction was applied. The linear absorption coefficient, μ, for Cu-Kα radiation is 9.0 cm^{-1}.

Structure solution and refinement: The structure was solved by direct methods and expanded using Fourier techniques. The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. All calculations were performed using the teXsan crystallographic software package of Molecular Structure Corporation.

Biological Tests:

Antifungal test: The method for determination of MIC (Minimum Inhibitory Concentration) of the compounds was mostly taken from recommendations of NCCLS (National Committee for Clinical Laboratory Standards). Compounds were dissolved in acetone, methanol or water (proper controls were put to cancel out the solvent effect in our calculations). Solutions of fractions or compounds were diluted in a two-fold manner in the medium (RPMI-1640, without adding bicarbonate), in 96-well plates, remaining 100 μl in each well. The highest concentration in each case was 1000 μg/ml. The fungal organisms included *Cryptococcus neoformans* KF-33, *Candida albicans* ATCC 14053, *Saccharomyces cerevisiae* PLM 454. The organisms were grown on SDA slants for 24-48 h at 30^0C. Then the fungi were suspended in the normal saline solution to obtain a T of 75%-77% at 530 nm, which is equal to 10^6 CFU/ml. The fungal suspension was diluted 200 times and 100 μl was added to each well. Incubation period was 24 h at 30^0C. Reading of the results was done by the help of an inverted microscope with a magnification of 40. Negative and positive controls, in which no test compound and a known drug like amphotericin B applied respectively, were used to have a comparison basis. The MIC was defined as the minimum concentration of the agent that shows a full inhibition of the fungus in the well, which was observed by the microscope.

cAMP-Phosphodiesterase inhibitory activity: Beef heart phosphodiesterase was used in this study. The effects on the enzyme activity were assayed by the method described in chapt. 6 (Nikkaido, *et al.*, 1981).

5-Lipoxygenase inhibitory activity: 5-Lipoxygenase from RBL-1 cells was used in this study. The effects on the enzyme activity were assayed by the method described before (Kimura, *et al.*, 1987). The cells were sonicated in a Sonifier Cell Disruptor (Bransor Sonic Power Co.) and the sonicated preparations (1.3×10^7 cells/tube) were preincubated with test compounds and 2.0 mM $CaCl_2$ for 5 min at 37°C. Then [1-^{14}C] arachidonic acid (0.1 μCi) was added at a final concentration of 1.67 nmol (144,560 cpm)/0.4 ml per tube, and the mixture was incubated for 5 min at 37°C. The reaction was stopped by adding 0.5 (N) HCOOH and the mixture was extracted with 8 volumes of EtOAc. The EtOAc phase was evaporated under N_2, and the residue was dissolved in a small amount of EtOAc phase (40 μl), applied to pre-coated silica gel TLC plastic sheets, and developed with Et_2O-petroleum ether-AcOH (50:50:1, v/v). Metabolites were identified by comparison with those of authentic samples. Radioactive spots were detected by autoradiography, cut out with scissors, and counted in a liquid scintillation counter.

Free radical scavenging activity: Each sample (0.025 mM) in EtOH (2ml) was added to a test tube containing 1 ml of 0.5 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) in EtOH and 2 ml of 0.1 M acetate buffer (pH 5.5). The reaction mixture was allowed to stand for 30 min at room temperature. The concentration of DPPH radical was measured by the absorbance at 517 nm. The mean effective concentrations of test samples, required to decrease the absorption by 0.2, were calculated and expressed as the $IC_{0.2}$ values (Pan, *et al.*, 1994; Cotelle, *et al.*, 1996).

Isolated compounds:

^1H- and ^{13}C-NMR spectra were taken at 400 and 100 MHz respectively; chemical shifts are given in δ (ppm) relative to TMS as an internal standard.

Verbascoside (Acteoside) (1) Pale yellow amorphous powder, mp 119-123^{0}C (uncorr.), $[\alpha]^{21}$D -65.46^0 (*c*= 0.34, MeOH). EI-MS m/z (%): 279 (11.4), 167 (37.2), 149 (100). CI-MS (methane as the ionizing gas) m/z (%): 429 (0.6), 391 (73.7). UV λ^{MeOH} nm (log ε): 206 (6.02) sh, 222 (5.04), 276 (4.39), 334.5 (4.08), 391.5 (3.75). IR ν (KBr) cm^{-1}: 3412 (OH), 2924 (aliphatic C-H), 1710 (α,β-unsaturated C=O), 1632, 1610, 1518 (arom. C=C), 1464, 1454 (aliphatic C-H), 1286 (alc. OH), 1156, 1036 (C-O), 1116, 1068 (C-O-C), 854, 814 (1,2,4 substit. of arom. group). ^{1}H-NMR (CD_3OD): δ 1.09 (6H, d, *J* =6 Hz, Rha H-6'), 2.78 (2H, m, H-7'), 3.26 (1H, m, Rha H-4'), 3.28 (1H, overlapping, Glc H-2), 3.37 (1H, m, Rha H-3'), 3.39 (1H, m, Rha H-5'), 3.50 (1H, m, Glc H-5), 3.52, 371 (2H, overlapping, Glc H-6), 3.75, 3.82 (2H, m, H-8'), 3.76 (1H, overlapping, Glc H-3), 3.91 (1H, dd, *J* = 1.7, 3.2 Hz, Rha H-2'), 4.37 (1H, d, *J* =7.5 Hz, Glc H-1), 4.89 (1H, t, *J* =9.2 Hz, Glc H-4), 5.18 (1H, d, *J* =1.4 Hz, Rha H-1), 6.26 (1H, d, *J* =16.1 Hz, H-8), 6.55 (1H, dd, *J* = 2, 7.8 Hz, H-6'), 6.67 (1H, d, *J* =7.8 Hz, H-5'), 6.69 (1H, d, *J* =2 Hz, H-2'), 6.77 (1H, d, *J* =8.3 Hz, H-5), 6.96 (1H, dd, *J* =2, 8.3 Hz, H-6), 7.04 (1H, d, *J* =2 Hz, H-2), 7.58 (1H, d, *J* =16.1 Hz, H-7). ^{13}C-NMR (see Table 4-1).

Syringin (2) White crystals, mp 186-188^{0}C (uncorr.), $[\alpha]^{25}$D -16.21^0 (*c*= 0.37, MeOH). EI-MS m/z (%): 210 (100),182 (34), 167 (38.1), 154 (20.4). CI-MS (methane as the ionizing gas) m/z (%): 211 (78.6), 210 (55.1), 193 (100). UV λ^{MeOH} nm (log ε): 221 (4.66), 266.5 (4.29). IR ν (KBr) cm^{-1}: 3388 (OH), 1590, 1512 (arom. C=C), 1422 (aliph. C-H), 1250, 1028 (C-O-C), 1134 (alc. C-O). ^{1}H-NMR (CD_3OD): δ 3.21 (1H, m, Glc H-5), 3.39 (1H, m, Glc H-3), 3.41 (1H, m, Glc H-4), 3.46 (1H, m, Glc H-2), 3.65 (1H, dd, *J* =5.4, 12 Hz, Glc H-6), 3.77 (1H, dd, *J* =2.4, 12 Hz, Glc H-6), 3.85 (9H, s, OMe), 4.21 (2H, dd, *J* =1.5, 5.8 Hz, H-1), 4.85 (1H, d, *J* =5.3 Hz, Glc H-1), 6.30, 6.34 (1H, dt, *J* =5.7, 15.6 Hz, H-2), 6.54 (1H, d, *J* =15.6 Hz, H-3), 6.7 (2H, s, H-2,6). ^{13}C-NMR (see Table 4-1). X-ray three-dimensional structural drawing with bond distances of syringin is shown in Figure 4-5.

Ligstroside (3) Pale yellow amorphous powder, mp 75-78^{0}C (uncorr.), $[\alpha]^{25}$D -120.87^0 (*c*= 0.37, MeOH). EI-MS m/z (%): 279 (12.4), 167 (34.3), 149 (100), 113 (12.7), 104

(7.7), 83 (7.8), 71 (24.9). FAB-MS *m/z*: 547.2 $[M+Na]^+$, 525.2 $[M+H]^+$. UV λ^{MeOH} nm (log ε): 202 (4.59), 226 (4.25), 277 (3.40). IR ν (KBr) cm^{-1}: 3471, 3448, 3428 (OH), 2952, 2928 (aliph. C-H), , 1728 (C=O), 1630 (conjugated enol ether, C=C), 1518 (arom. C=C), 1442 (aliph. C-H), 1390 (alc. O-H), 1274, 1160, 1076, 1046 (C-O),. ^{1}H-NMR (CD_3OD): δ 1.60 (3H, dd, *J* = 1.2, 7.1 Hz, H-10), 2.38 (1H, dd, *J* =9.3, 14.2 Hz, H-6a), 2.66 (1H, dd, *J* =4.4, 14.2 Hz, H-6b), 2.77 (2H, t, *J* =6.8 Hz, H-2'), 3.26-3.28 (3H, m, Glc H-2,4,5), 3.4 (1H, t, *J* =8.8 Hz, Glc H-3), 3.57 (1H, dd, *J* =5.4, 11.7 Hz, Glc H-6a), 3.66 (1H, s, OMe), 3.82 (1H, dd, *J* =1.5, 11.7 Hz, Glc H-6b), 3.92 (1H, dd, *J* =4.4, 9.3 Hz, H-5), 4.06 (1H, dt, *J* =6.8, 10.7 Hz, H-1'b), 4.18 (1H, dt, *J* =6.8, 10.7 Hz, H-1'a), 4.78 (1H,d, *J* =7.8 Hz, Glc H-1), 5.87 (1H, brs, H-1), 6.03 (1H,q, *J* =6.8 Hz, H-8), 6.68 (2H, d, *J* =8.3 Hz, H-5',7'), 7.0 (2H, d, *J* =8.3 Hz, H-4',8'), 7.46 (1H, s, H-3). ^{13}C-NMR (see Table 4-1).

10-hydroxyligstroside (4) Pale yellow amorphous powder, mp 88-90^{0}C (uncorr.), $[\alpha]^{24}D$ -140.86^0 (*c*= 0.38, MeOH). EI-MS m/z (%): 250 (0.6), 138 (28.3), 120 (7.8), 107 (100), 77 (21.3). FAB-MS *m/z*: 563.2 $[M+Na]^+$, 541.3 $[M+H]^+$. UV λ^{MeOH} nm (log ε): 202 (4.42) sh, 227 (4.28), 278 (3.38). IR ν (KBr) cm^{-1}: 3436 (OH), 2952, 2916 (aliph. C-H), 1712 (C=O), 1632 (conjugated enol ether, C=C), 1596, 1518 (arom. C=C), 1454 (aliph. C-H), 1392, 1358, 1346, 1304 (alc. O-H), 1162, 1106, 1080, 1044 (C-O). ^{1}H-NMR (CD_3OD): δ 2.48 (1H, dd, *J* =9.3, 14.9 Hz, H-6b), 2.72 (1H, dd, *J* = 4.4, 14.9 Hz), 2.81 (2H, t, *J* =7.1 Hz, H-2'), 3.29-3.33 (3H, overlapping, Glc H-2,3,4), 3.42 (1H, t, *J* =8.8 Hz, Glc H-5), 3.66 (1H, dd, *J* =5.9, 12.2 Hz, Glc H-6a), 3.69 (3H, s, OMe), 3.88 (1H, dd, *J* =1.5, 11.7 Hz, Glu H-6b), 3.94 (1H, dd, *J* =4.4, 9.3 Hz, H-5), 4.15, 4.13 (2H, m, H-1'a,b), 4.26 (2H, m, H-10), 4.81 (1H, d, *J* =7.3 Hz, Glc H-1), 5.95 (1H, s, H-1), 6.13 (1H, t, *J* =6.3 Hz, H-8), 6.71 (2H, d, *J* =8.3 Hz, H-5',7'), 7.03 (2H, d, *J* =8.3 Hz, H-4',8'), 7.51 (1H, s, H-3). ^{13}C-NMR (see Table 4-1).

Table 4-1. ^{13}C NMR spectral data for verbascoside, syringin, ligstroside and 10-OH ligstroside (in CD_3OD, 100MHz).

Carbon	Verbascoside	Syringin	ligstroside	10-OH ligstroside
1	127.9		95.2	94.6
2	115.4			
3	147.0		155.1	155.0
4	150.0		109.5	109.2
5	116.7		31.8	32.3
6	123.4		41.3	41.2
7	148.2		173.2	173.0
8	114.9		124.9	129.4
9	168.5		130.5	129.9
10			13.5	59.2
11			168.7	168.4
1'	131.7	135.5	66.9	66.9
2'	117.3	105.6	35.2	35.1
3'	146.3	154.5	130.0	131.0
4'	144.9	136.1	131.0	130.9
5'	116.5	154.5	116.3	116.3
6'	121.5	105.6	156.9	157.0
7'	36.8	131.5	116.3	116.3
8'	72.6	130.2	131.0	130.9
9'		63.8		
Glc-1	104.4	105.5	100.9	100.9
Glc-2	76.3	75.9	74.8	74.7
Glc-3	81.9	78.0[a]	77.9	78.4
Glc-4	70.6	71.5[a]	71.5	71.5
Glc-5	76.4	78.6	78.5	77.9
Glc-6	62.5	62.8	62.8	62.8
Rha-1	103.2			
Rha-2'	72.5			
Rha-3'	72.3			
Rha-4'	74.0			
Rha-5'	70.8			
Rha-6'	18.7			
OMe		57.2	51.9	51.9

a. Assignment might be interchangeable (within each column).

Table 4-2. Bond length (Å) in syringin molecule, based on X-ray crystallographic data.

atom	Atom	distance	atom	Atom	distance
O(1)	C(3)	1.354(6)	O(1)	C(16)	1.428(7)
O(2)	C(4)	1.387(5)	O(2)	C(10)	1.414(6)
O(3)	C(5)	1.348(7)	O(3)	C(17)	1.407(8)
O(4)	C(9)	1.406(10)	O(4)	H(4O)	0.83
O(5)	C(11)	1.423(6)	O(5)	H(5O)	0.96
O(6)	C(12)	1.421(6)	O(6)	H(6O)	0.98
O(7)	C(13)	1.411(7)	O(7)	H(7O)	0.98
O(8)	C(10)	1.408(5)	O(8)	C(14)	1.438(7)
O(9)	C(15)	1.431(7)	O(9)	H(9O)	0.95
O(10)	H(10Ob)	0.70	O(10)	H(10Oa)	1.01
C(1)	C(2)	1.373(8)	C(1)	C(6)	1.390(8)
C(1)	C(7)	1.498(8)	C(2)	C(3)	1.403(7)
C(2)	H(2)	1.08	C(3)	C(4)	1.394(8)
C(4)	C(5)	1.382(8)	C(5)	C(6)	1.409(7)
C(6)	H(6)	1.14	C(7)	C(8)	1.28(1)
C(7)	H(7)	1.00	C(8)	C(9)	1.513(9)
C(8)	H(8)	0.96	C(9)	H(9b)	0.97
C(9)	H(9a)	0.97	C(10)	C(11)	1.530(7)
C(10)	H(10)	1.02	C(11)	C(12)	1.489(8)
C(11)	H(11)	0.99	C(12)	C(13)	1.524(7)
C(12)	H(12)	0.99	C(13)	C(14)	1.533(7)
C(13)	H(13)	1.10	C(14)	C(15)	1.499(7)
C(14)	H(14)	0.97	C(15)	H(15a)	1.16
C(15)	H(15b)	1.06	C(16)	H(16a)	0.92
C(16)	H(16b)	0.97	C(16)	H(16c)	1.02
C(17)	H(17a)	0.97	C(17)	H(17b)	0.93
C(17)	H(17c)	0.97			

Results

As a search for understanding of the components of *F. americana*, collection of the branches of this plant was completed in Canada. Extraction of the bark material was performed with methanol. Following fractionation of the total extract with organic solvents, chromatographic separation using Sephadex LH-20, guided by TLC and HPLC was started on EtOAc fraction. The result was separation of compounds **1**, **3** and **4**. Syringin (**2**) was crystallized directly from n-BuOH fraction.

The spectral data of verbascoside, syringin, ligstroside, and 10-hydroxyligstroside are well in agreement with the authentic samples and the literature (Andary, *et al.*, 1982) verbascoside; (Shen, *et al.*, 1990) 10-hydroxyligstroside; (El-Naggar, *et al.*, 1980; LaLonde, *et al.*, 1976) Ligstroside and 10-hydroxyligstroside; (Tanahashi, *et al.*, 1992) ligstroside (Fig. 4-3).

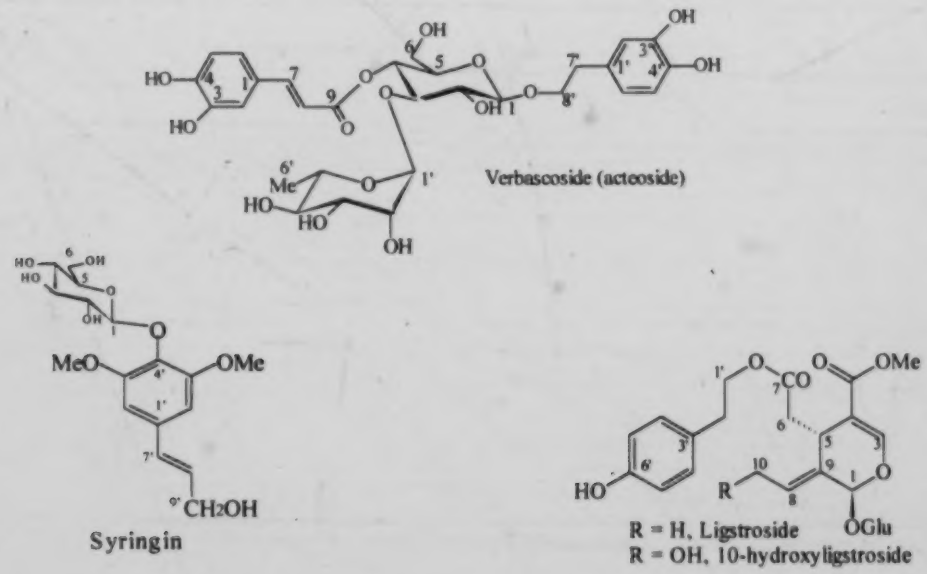


Figure 4-3. Structures of the compounds isolated from *Fraxinus americana*.

Other structural possibilities were ruled out by comparing the spectral data. These include the following: Formoside which has a tyrosyl group with esterified phenolic hydroxyl, instead of alcoholic hydroxyl in ligstroside (Tanahashi, *et al.*, 1993); isoligstroside which has the esterified tyrosyl and methyl in the place of the other one compared to ligstroside (Tanahashi, *et al.*, 1992; Zheng-Dan He, *et al.*, 1993). Also, NOESY spectroscopy of ligstroside showed an interaction between OMe hydrogens and H-3 plus other important interactions showed in Fig. 4-4.

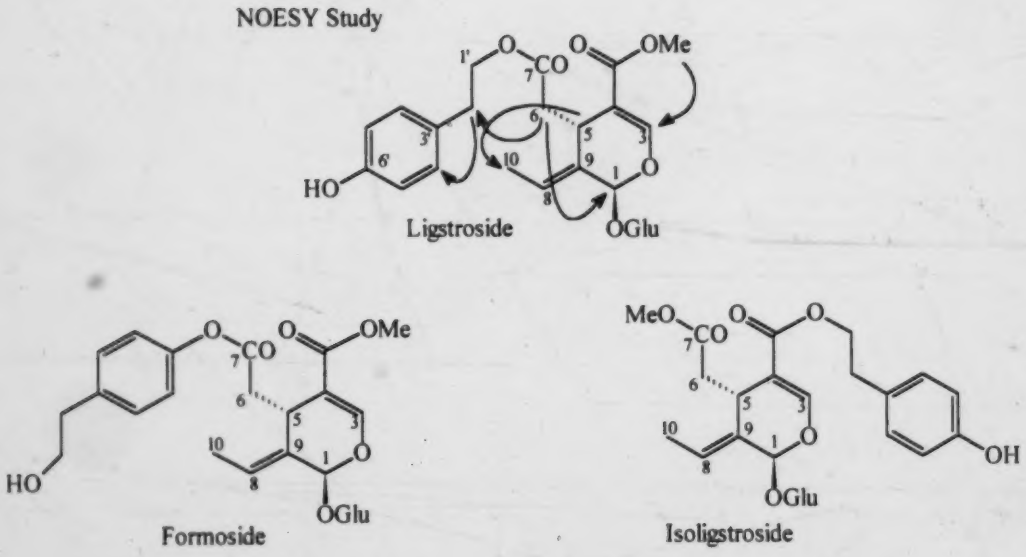


Figure 4-4. Result of NOESY spectroscopy of ligstroside that rules out the other structural possibilities such as formoside and isoligstroside.

In the case of compound (**2**) the physical and spectroscopic values were compared with those of literature and authentic sample and matched them clearly. (Sutarjadi, *et al.*, 1978). Furthermore, the X-ray crystallographic data were obtained for this compound. The detailed bond distances and the three dimensional structure of syringin crystal are shown in Table 4-2 and Figures 4-6 and 4-7 respectively.

Antifungal susceptibility testing of the *F. americana* bark extract fractions and compounds **1-4**, was performed and the results are shown in Table 4-3. As is shown, only a weak activity is presented by ligstroside and fraction P, while the rest are practically inactive.

Table 4-3. Antifungal activities, MIC (μg/ml) of *F. americana* fractions and compounds 1-4.

Fraction or Compound	*C. neoformans*[a]	*C. albicans*[b]	*S. cerevisiae*[c]
ET[d]	-	-	nt[e]
EA	-	-	nt
Bu	-	-	nt
Aq	-	-	nt
P	500	-	nt
1(Verbascoside)	1000	-	-
2(Syringin)	-	-	-
3(Ligstroside)	500	1000	-
4(10-hydroxyligstroside)	-	-	-
Amphotericin B	0.8	0.8	0.4

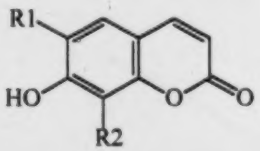
[a] *Cryptococcus neoformans* KF-33, [b] *Candida albicans* ATCC 14053, [c] *Saccharomyces cerevisiae* PLM 454, [d] Abbreviations: ET, ether fraction; EA, ethyl acetate fraction; Bu, n-butanol fraction; Aq, water fraction; P, precipitate from the total extract by addition of water. [e] Not tested

In regard to the other biological activities, the cAMP-phosphodiesterase inhibitory activity (PDI) of the isolated compounds were measured and the results are shown in Table 4-4. PDI activity of coumarins, esculin, esculetin, fraxin and fraxetin have also been reported here. PDI activity screening test is a useful tool for detecting the biologically active compounds in natural medicines (Nikaido, *et al.*, 1981). In the present study, the cAMP-phosphodiesterase inhibitory activity of compounds from *F. americana* are indicated not to be comparable with coumarins, such as esculetin and fraxin, which are usually found in other species of *Fraxinus* (Table 4-4). The 5-lipoxygenase inhibitory activity, which is related to anti-inflammatory effect, has been shown in Table 4-4 as well. In this regard, most of the coumarins tested, syringin and iridoids did not show any activity. However, verbascoside (acteoside) and esculetin indicated potent inhibitory activity. Antioxidative property is another bioactivity studied here. Verbascoside and esculetin were found to be the two most potent radical scavengers followed by fraxin.

Table 4-4. Enzyme inhibitory and antioxidant activities of compounds from *F. americana* bark and several coumarins.

Compound	cAMP Phosphodiesterase[a]	5-Lipoxygenase[b]	DPPH[c]
Verbascoside	>50	1.4	3.4
10-Hydroxyligstroside	>50	>50	153.9
Ligstroside	>50	>50	80.3
Syringin	>50	>50	95.2
Esculetin	19.7	7.9	3.8
Esculin	>50	>50	>100
Fraxetin	>50	>50	>100
Fraxin	9.0	>50	31.2

[a] IC_{50} ($\times 10^{-5}$ M); [b] IC_{50} ($\times 10^{-6}$ M); [c] $IC_{0.2}$ ($\times 10^{-6}$ M)



Esculin: R1 = OGlu, R2 = H
Esculetin: R1 = OH, R2 = H
Fraxin: R1 = OMe, R2 = OGlu
Fraxetin: R1 = OMe, R2 = OH

Figure 4-5. Structure of coumarins used in the enzyme inhibitory and radical scavenging activity tests.

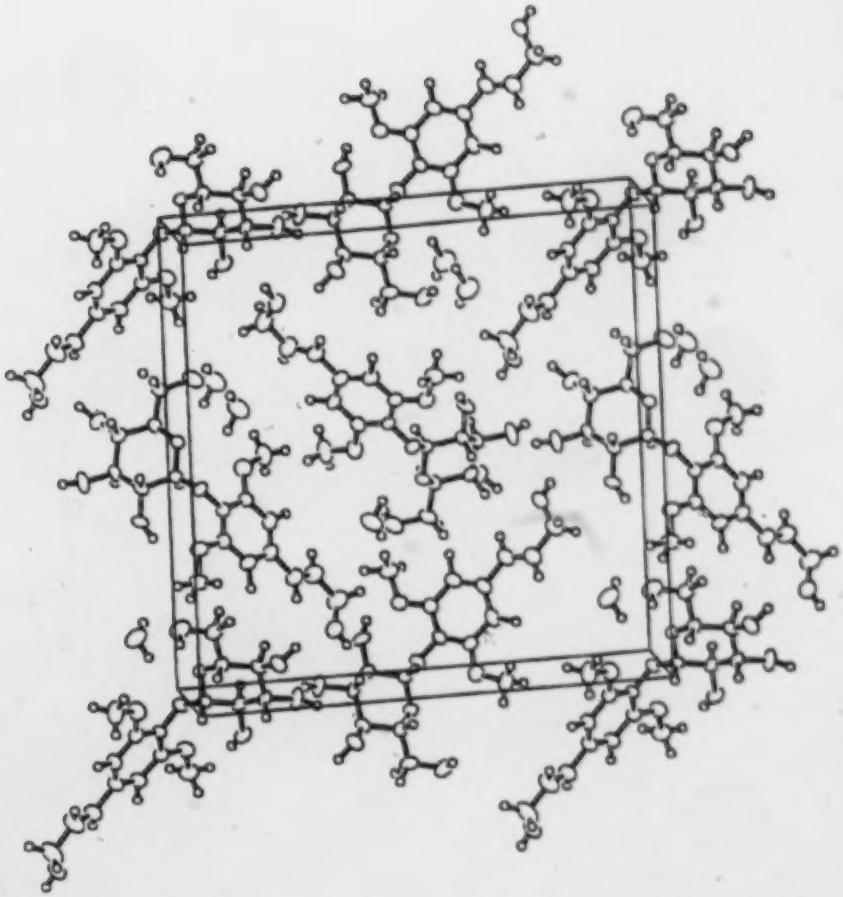


Figure 4-6. X-ray three-dimensional structure of syringin crystal.

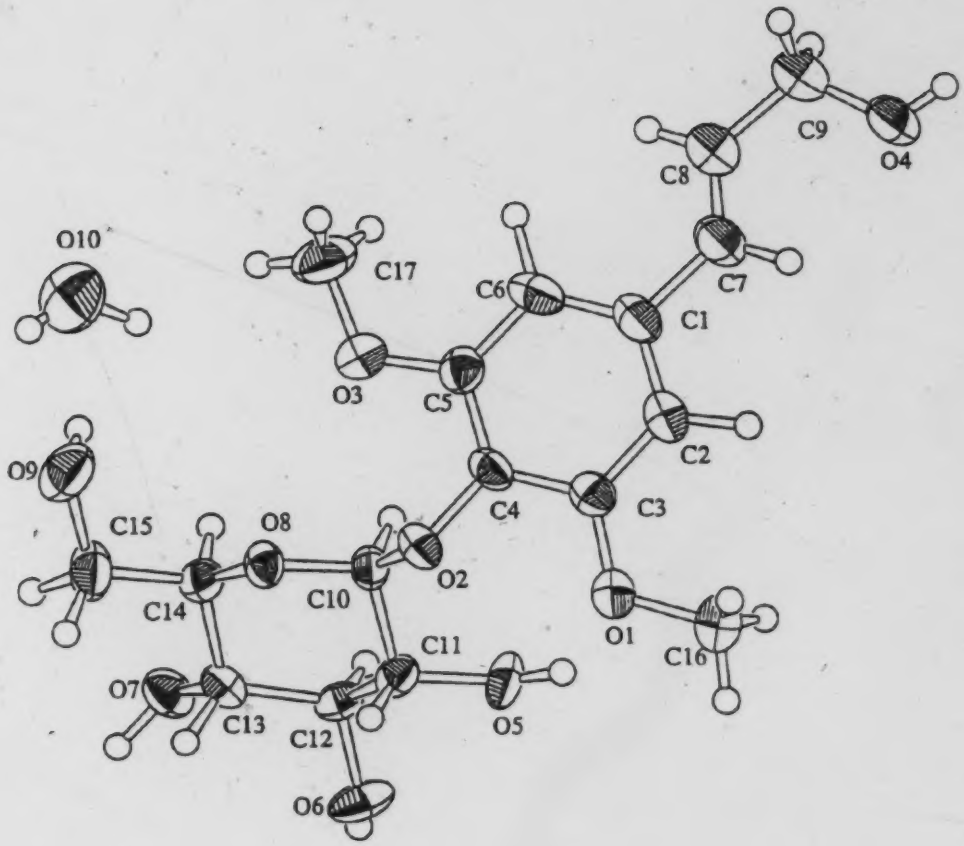


Figure 4-7. X-ray three-dimensional structure of syringin molecule. In each case, bond distances are shown in table 4-2.

Discussion

Verbascoside has been reported to have immunosuppressive (Sasaki, *et al.*, 1989), anti-inflammatory (Murai, *et al.*, 1995), and antinephritic (Hayashi, *et al.*, 1994) effects *in vivo* and to be one of the major constituents in natural medicines used as diuretics such as *Plantago* seed (Kawamura, *et al.*, 1997), *Forsythia* fruit (Noro, *et al.* 1991), *Rehmannia* root (Sasaki, *et al.*, 1989) and so on.

Syringin has been reported to have anti-stress effects (Takasugi, *et al.*, 1985) *in vivo* and to be one of the major constituents of natural medicines used as tonics such as *Eleutherococcus* (Siberian ginseng) (Nishibe, *et al.*, 1990), and Cortex Eucommiae (Oshima, *et al.*, 1988; Deyama, *et al.*, 1983). Therefore, verbascoside and syringin could be responsible for the reported activities of *Fraxinus americana* and justify the traditional usage of the bark among North American Natives.

In a report, antibacterial and antifungal activities of *Fraxinus ornus* bark has been mentioned (Kostova, *et al.*, 1993). The ethyl acetate fraction of the bark showed the highest activity against *Staphylococcus aureus* and *Candida* sp. This extract was reported to be rich in esculin, fraxin and fraxetin. The mentioned findings support the traditional usage of *Fraxinus* bark in folk medicine as a wound-healing agent. Also, the bark of *F. japonica* has been investigated for bioactive compounds, which ended in finding of esculin (Kodaira, *et al.*, 1981). In addition, amounts of esculin and esculetin in the bark of 19 species of *Fraxinus* from China were measured (Jialin, *et al.*, 1983). Among these species, the barks of 9 species are used in the treatment of dysentery. Fraxin has presumably been considered as one of the constituents of *F. americana* bark. (Millspaugh, 1974). But by using HPLC and comparing with standards, no sign of fraxetin, fraxin, esculin, and esculetin was found in the bark in our study; instead, isolation of the major components guided us to the isolation of **1-4**. The lack of antifungal activity of the tested fractions, as shown in Table 4-3, and the absence of the searched

coumarins in *F. americana* bark extract could lead to this conclusion that the observed antimicrobial activities in other *Fraxinus* sp. could be due to their coumarins.

We could not attribute any strong antifungal activity against the tested pathogens to the isolated iridoids and phenylethanoid. However, a number of acylated secoiridoid glycoside has been reported to be isolated from Chinese traditional drugs with usage in the treatment of infectious diseases. (Ma, *et al.*, 1994). Oleuropein, a compound structurally related to ligstroside, has been referred to as a phytoalexin precursor in the defense mechanism of *Olea europea* (Kubo, *et al.*, 1985).

As reported earlier, ligstroside, isolated from the bark, of *Fraxinus ornus* has shown relatively strong antibacterial effects. Tyrosol itself was not significantly active against the tested organisms. The report states an antifungal activity for ligstroside and some other secoiridoids against *Cladosporium cucumerinum*, a plant pathogen (Iossifova, *et al.*, 1994). Regarding our antifungal testing results (Table 4-3), the idea of antifungal activity for ligstroside against human opportunistic fungi is supported. Compound **4** with an extra hydroxyl, however, is not active in this test. The reason for this case might be a subject of further research.

One of the natural compounds close to verbascoside is orobanchoside with an extra OH on C-7' and a shift of rhamnose attachment to position 2 of glucose. Orobanchoside is reported to have antifungal activities against plant pathogenic fungi (Harborne, *et al.*, 1993). On the other hand, esters and amide derivatives of caffeic acid are believed to be part of the chemical defense system used by plants against pathogenic attack especially in hypersensitive responses (Legrand, *et al.*, 1976). However, our results do not indicate any antifungal activity for verbascoside. In this regard, and considering the reported anticancer property of verbascoside (Saracoglu, *et al.*, 1995), more attention should be paid to the mechanism of specific action of verbascoside against neoplastic cells. This finding may lead to new targets in cancer research.

The inhibition of dihydrofolate reductase (DHFR) is correlated with cytostatic and thus antibacterial effects (Strehl, *et al.*, 1995). In this case, verbascoside might be effective on this target. Also a correlation between anti-inflammatory activities and DHFR inhibition has been reported. It was concluded that antifolate and antiprostaglandin activities might cooperate in the entire anti-inflammatory effects. *F. excelsior* with an apparent IC_{50}-value of 0.008% (w/v) by far dominates the overall DHFR inhibitory effect of the combination of *F. excelsior*, *Populus tremula* and *Solidago virgaurea* (Strehl, *et al.*, 1995; Kruedener, *et al.*, 1995). Observing the strong lipoxygenase inhibitory property of verbascoside can explain the traditional usage of *F. americana* on sores and for snakebites.

Reactive oxygen species such as superoxide radical anion are involved in several diseases such as inflammatory processes, arthritis, arteriosclerosis, cataract and others. The oxidative coupling has been suggested to play a role in deactivating of viral particles. (Pierpoint, *et al.*, 1977). Many antioxidants were found to inhibit different fungal and bacterial species (Branen, *et al.*, 1997). Components of the phytomedicine Phytodolor N, containing the extract of *F. excelsior* and other components, possess antipyretic, analgesic, anti-inflammatory and antirheumatic activity. This herbal drug along with its components shows radical scavenging properties. In addition, although usage of *F. americana* bark has been described to be similar to the usage of *F. japonica* and *F. excelsior* bark in Asia and Europe (Hansel, 1994), the coumarin constituents were not detected in *F. americana*. This finding urges a reinvestigation on the *in vivo* effects of this product.

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Chapter 5

Synthesis and Antifungal Activity of Coumarins

Introduction

The active area of drug discovery and development is very much a multidisciplinary field. The competition among scientists is growing to access new resources and better approaches to find new cures for different diseases. In the antimicrobial field of research an additional force is pushing this ongoing effort further, which is the emergence of new infectious diseases and resistance of the previously susceptible organisms to the existing drugs.

Emergence of new resistant species of fungi in addition to the poor safety and pharmacokinetic profile has challenged clinicians in their approach to treat fungal infections. Combination therapy, improving delivery systems, immunotherapy, synthesis of new analogues of the present antifungal drugs, and finally discovery of new prototypes are among the possible methods to improve antifungal therapy, in general.

Natural products can be considered as a rich source of diverse molecules to produce new generations of antifungal compounds. There are many antifungal compounds of plant origin. These compounds may be constitutive, which present in plant tissue most of the time; or induced, that are produced in plants only in special circumstances like infection. In our efforts to study natural sources for antifungal agents, in the previous chapters we started with screening a group of selected plants and their high percentage of active extracts encouraged us in our further studies. According to chapter 3, four coumarins were obtained through a bioassay-guided isolation of active components from *Diplotaenia damavandica*, a rare Iranian native plant. Coumarins, which show diverse biological activities, can be classified as one of the defense-designed group of plant secondary metabolites (Grayer, *et al.*, 1994). The coumarin skeleton of angelicin **(28)**, which was the most active compound isolated from the plant, was considered as the lead structure in our study. To improve the potency and antifungal profile of the lead structure, different modifications were considered. Based on the strategy of "mimicry mixed

functionality", several derivatives of angelicin were designed and synthesized. The antifungal activities of those synthesized coumarins and angelicin derivatives will be discussed.

Materials and Methods

Melting points were determined on a Fischer melting point apparatus and are uncorrected. The ^{1}H-NMR spectra were recorded on either a JEOL JNM-GX270 or Brucker AM-300 spectrometer, using tetramethylsilane as an internal standard. High-resolution mass spectra were determined on an AEI MS 50 spectrometer equipped with a Mass Spectrometry Services MASPEC data system. IR data were recorded on a NICOLET Magna 750 FT IR instrument equipped with a NICPLAN microscope attachment. Microanalyses were performed by using an EA 1108-Elemental Analyzer, Carlo Erba Instruments and were within ± 0.4% of the theoretical values for all elements listed. Silica gel column chromatography was carried out using Merck 7734 (60-200 mesh) silica gel. Physicochemical properties were estimated using the PALLAS computational program. The Prolog P module was used to calculate the log P values and the Prolog D 2.0 module was used to predict the logarithm of distribution coefficient (PALLAS, 1995). Since the medium used for fungal susceptibility test, is adjusted to pH=7, the log $D_{7.0}$ values are calculated in this paper. The solid compounds synthesized here are referred to as powder (amorphous), unless otherwise mentioned to be crystalline.

Compounds 1-3 These compounds were kindly provided by Dr. T. Harayama, which were reported to be synthesized previously (Harayama, *et al.*, 1996).

7-[(2-Propynoyl)oxy]-2*H*-1-benzopyran-2-one (23) A mixture of propiolic acid (20 µl, 0.3 mmol), and DCC (dicyclohexyl carbodiimide) (60 mg, 0.3 mmol), in CH_2Cl_2 (5 ml), was stirred for 30 min. at 0 ^{0}C and then umbelliferone, **21**, (50 mg, 0.3 mmol) was added. After 24 h stirring at room temperature, the mixture was filtered. The filtrate was dried and washed with *n*-pentane and then hexane:ether (1:1) to remove the urea derivative. The left over was subjected to chromatography (CH_2Cl_2) to afford **23** (22 mg,

0.1 mmol, 34%) as a white powder, mp 153-157 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.63 (1H, d, *J*= 9.5), 7.41 (1H, d, *J*= 8.4), 7.16 (1H, d, *J*= 2.2), 7.10 (1H, dd, *J*= 8.4, 2.2), 6.38 (1H, d, *J*= 9.7), 3.26 (1H, s). ^{13}C-NMR ($CDCl_3$ + CD_3OD) δ: 163.4, 157.2, 154.9, 152.8, 146.1, 131.8, 120.6, 120.1, 118.3, 112.4, 74.2, 30.9. IR (KBr): 3240, 2132, 1734, 1624, 1178, 1124 cm^{-1}. HR MS m/z: 214.02615 (M^+) (Calcd. for $C_{12}H_6O_4$: 214.02661).

7-(2-Propynoxy)-2*H*-1-benzopyran-2-one (24) A mixture of 1-bromo-2-propyne (160 μl, 1.8 mmol), and **21** (200 mg, 1.2 mmol) in dry DMF containing K_2CO_3 (225 mg, 1.3 mmol) was stirred for 10 h at room temperature. After the addition of $CHCl_3$ and H_2O (20 ml each), the aqueous phase was extracted two more times with $CHCl_3$ and the organic phase was washed three times with water (10 ml each). Chromatography of the dried organic phase (hexane: ether, 1:1) afforded **24** (235 mg, 1.15 mmol, 96%) as a pale yellow powder, mp 110-112 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.66 (1H, d, *J*= 9.5), 7.42 (1H, d, *J*= 8.5), 6.96 (1H, d, *J*= 2.4), 6.93 (1H, dd, *J*= 2.4, 8.2), 6.29 (1H, d, *J*= 9.5), 4.78 (2H, d, *J*= 2.4), 2.59 (1H, t, *J*= 2.4). IR ($CHCl_3$ cast): 3265, 3084, 2119, 1724, 1616 cm^{-1}. HR MS m/z: 200.04815 (M^+) (Calcd. for $C_{12}H_8O_3$: 200.04735).

7-[[4-(Trifluoromethyl)benzoyl]oxy]-2*H*-1-benzopyran-2-one (25) A mixture of 21 (30 mg, 0.2 mmol), *p*-(trifluromethyl)benzoyl chloride (63 mg, 0.3 mmol), DMAP (catalytic amount), and NEt_3 (20 mg, 0.2 mmol) in dry THF (5ml) was stirred for 2 h at room temperature. Then water and chloroform (20 ml each) were added and the aqueous layer was extracted two more times with $CHCl_3$. The organic phase was washed three times with HCl (0.1 N, 20 ml portions) and chromatographed (CH_2Cl_2) to afford **25** (63 mg, 0.19 mmol, 95%) as a white powder, mp 175-176 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 8.35 (2H, d, *J*= 8.3), 7.83 (2H, d, *J*= 8.3), 7.75 (1H, d, *J*= 9.5), 7.59 (1H, d, *J*= 8.5), 7.28 (1H, d, *J*= 2.3), 7.21 (1H, dd, *J*= 2.3, 8.6), 6.46 (1H, d, *J*= 9.6). ^{13}C-NMR ($CDCl_3$) δ: 163.3, 160.1, 154.8, 153.0, 142.6, 135.8, 135.2, 132.0, 130.7 (2C), 128.7, 125.7, 118.3, 117.0, 116.4 (2C), 110.5. IR ($CHCl_3$ cast): 1732, 1701, 1329, 1128, 1065 cm^{-1}. HR MS m/z: 334.04611 (M^+) (Calcd. for $C_{17}F_3H_9O_4$: 334.04529).

2*H*-Furo[2,3-*h*]-1-benzopyran-2-one-8-carboxylic acid (27) Compound **26** (50 mg, 0.2 mmol), was mixed with 5 ml of aqueous NaOH (20%) and the mixture was refluxed for 24 h. After cooling and acidification with conc. HCl, the mixture was vigorously stirred for 1 h and the precipitate collected by filtration and washed with $CHCl_3$, EtOAc, MeOH and water (20 ml each) to afford **27** (40 mg, 0.17 mmol, 87%) as a white amorphous solid, mp >305 ^{0}C (decomposed). ^{1}H-NMR (DMSO-d_6) δ: 13.86 (1H, bs), 8.21 (1H, d, *J*=9.7), 7.86 (1H, s), 7.84 (1H, d, *J*= 8.6), 7.73 (1H, d, *J*=8.5), 6.52 (1H, d, *J*=9.7). ^{13}C-NMR (DMSO-d_6) δ: 159.5, 156.8, 148.6, 147.3, 145.1, 127.7, 121.2, 116.0, 114.4, 114.1, 109.8, 109.3. IR (microscope): 3611, 3080, 1750, 1710, 1562 cm^{-1}. HR MS m/z: 230.02109 (M^+) (Calcd. for $C_{12}H_6O_5$: 230.02153).

8-(1-Oxo-3-bytynyl)-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (29) This compound was prepared from **27** (100 mg, 0.4 mmol) by a procedure similar to that explained for **24**, afforded 21 mg (0.08 mmol, 22%) as a yellow powder, mp 108-110 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.91 (1H, d, *J*= 0.6), 7.83 (1H, d, *J*= 9.8), 7.57 (1H, d, *J*= 8.5), 7.53 (1H, dd, *J*= 0.6, 8.5), 6.46 (1H, d, *J*= 9.5), 5.01 (2H, d, *J*= 2.4), 2.59 (1H, t, *J*= 2.4). IR ($CHCl_3$ cast): 3290, 2923, 2126, 1724 cm^{-1}. HR MS m/z: 268.03923 (M^+) (Calcd. for $C_{15}H_8O_5$: 268.03717).

8-Carbamoyl[*N*-[2-[ethyl(S)-4-methylpentanoate]]]-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (31) The method described earlier for peptide synthesis was used (Bodanszky, *et al.*, 1988). Compound **27** (690 mg, 3 mmol), L-leucine HCl (584 mg, 3 mmol), 1-hydroxybenzotriazol hydrate (405 mg, 3 mmol), and N-methylmorpholine (304 mg, 3mmol) were dissolved in dry THF (10 ml). The mixture was stirred in an ice-water bath while DCC (650 mg, 3 mmol) was being added. Stirring was continued for 1.5 h at 0 ^{o}C and another hour at room temperature. After filtration, EtOAc (100 ml) was added to the filtrate, then washed with a saturated solution of $NaHCO_3$ (twice, 25 ml each), citric acid solution (10%) (twice, 25 ml each) and finally with water (twice, 25 ml each), successively. Evaporation of the solvent afforded **31** (969 mg, 2.6 mmol, 87%) as a light brown powder, mp 151-154 ^{0}C. ^{1}H-NMR (DMSO-d_6) δ: 9.08 (1H, d, *J*=7.8), 8.21 (1H, d, *J*= 9.6), 7.94 (1H, s), 7.83 (1H, d, *J*= 8.6), 7.70 (1H, d, *J*= 8.8), 6.53 (1H, d, *J*= 9.5), 4.52

(1H, m), 4.13 (2H, q, *J*= 7.1), 1.67 (3H, m), 1.21 (3H, t, *J*= 7.1), 0.95 (3H, d, *J*= 6), 0.90 (3H, d, *J*=6). ^{13}C-NMR (DMSO-d_6) δ: 172.0, 159.5, 157.7, 156.2, 149.2, 148.4, 145.1, 127.1, 116.1, 114.3, 114.1, 109.0, 106.5, 60.7, 50.7, 24.4, 22.8, 21.1 (2C), 14.1. IR (microscope): 3332, 2957, 1736, 1673, 1522 cm^{-1}. HR MS m/z: 371.13632 (M^+) (Calcd for $C_{20}H_{21}NO_6$: 371.13690). Anal: C, 64.65%, H, 5.95% (Calcd. for $C_{20}H_{21}NO_6$: C 64.70%, H 5.66%).

8-Carbamoyl[*N*-[2-[(S)-4-methylpentanoic acid]]]-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (32) Compound **31** (50 mg, 0.1 mmol) was dissolved in NaOH (20 mg, in 10 ml water) and heated at 30-40 ^{0}C for 24 h. After washing the mixture with $CHCl_3$, HCl (1 N) was added. The mixture was filtered and the precipitate washed with water, then dissolved in acetone. The acetone solution was evaporated to afford **32** (17 mg, 0.05 mmol, 49%) as an orange powder, mp 220-223 ^{0}C. ^{1}H-NMR (DMSO-d_6) δ: 12.25 (1H, bs), 8.97 (1H, d, *J*=7.9), 8.22 (1H, d, *J*=9.5), 7.94 (1H, s), 7.82 (1H, d, *J*=8.7), 7.71 (1H, d, *J*=8.6), 6.52 (1H, d, *J*=9.5), 4.46 (1H, m), 1.76 (3H, m), 0.93 (3H, d, *J*=5.9), 0.89 (3H, d, *J*=5.9). ^{13}C-NMR (DMSO-d_6) δ: 173.6, 159.5, 157.6, 156.2, 149.5, 148.4, 145.1, 127.1, 116.1, 114.3, 114.1, 109.1, 106.3, 50.5, 24.5, 22.9, 21.1 (2C). HR MS m/z: 343.10586 (M^+) (Calcd. for $C_{18}H_{17}NO_6$: 343.10693).

8-Carbamoyl[*N*-[2-[ethyl(S)-3-phenylpropanoate]]]-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (33) This compound was prepared according to the same method described for **31**. Yield: 758 mg (1.9 mmol, 62%) as a pale yellow powder, mp 124-126 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.80 (1H, d, *J*= 9.7), 7.77 (1H, d, *J*= 0.7), 7.51 (1H, d, *J*= 8.6), 7.42 (1H, d, *J*= 9.0), 7.28 (3H, m), 7.17 (2H, m), 7.08 (1H, d, *J*= 7.9), 6.43 (1H, d, *J*= 9.7), 5.08 (1H, m), 4.23 (2H, q, *J*= 7.2), 3.27 (2H, d, *J*= 5.8), 1.28 (3H, t, *J*= 7.2). IR ($CHCl_3$ cast): 3328, 3088, 2932, 1739, 1672 cm^{-1}. HR MS m/z: 405.12222 (M^+) (Calcd. for $C_{23}H_{19}NO_6$: 405.40700).

8-Carbamoyl[*N*-[2-[(S)-3-phenylpropanoic acid]]]-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (34) Prepared from **33** (50 mg, 0.12 mmol) using the same method as described for **32**. Yield: 12 mg (0.03 mmol, 27%) as a yellow powder, mp 205-210 ^{0}C.

8-Carbamoyl[*N*-[6-[methyl-2-*N*-carbobenzoxy[(S)-2-aminohexanoate]]]]-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (35) As described for **31**, this compound was prepared from **27** (230 mg, 1 mmol). Yield: 500 mg (0.9 mmol, 98%) as a yellow powder, mp 114-115 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.80 (1H, d, *J*=9.6), 7.75 (1H, d, *J*=0.7), 7.50 (1H, d, *J*=8.6), 7.42 (1H, d, *J*=8.2), 7.30 (5H, m), 6.70 (1H, m), 6.43 (1H, d, *J*=9.5), 5.41(1H, d, *J*=8.1), 5.10 (2H, s), 4.40 (1H, m), 3.75 (3H, s), 3.49 (2H, m), 1.70 (4H, m), 1.48 (2H, m). IR ($CHCl_3$ cast): 3319, 1737, 1722, 1525 cm^{-1}. HRMS m/z: (M^+) 506.16917 (Calcd. for $C_{27}H_{26}N_2O_8$: 506.16891).

8-Carbamoyl[*N*-[6-[2-*N*-carbobenzoxy[(S)-2-aminohexanoic acid]]]]-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (36) As described for **32**, this compound was prepared from **35** (50 mg, 0.1 mmol) Yield: 10 mg (0.02 mmol, 20%) as a yellow powder. ^{1}H-NMR ($CDCl_3$) δ: 12.33 (1H, bs), 7.7 (1H, d, *J*= 9.5), 7.70 (1H, s), 7.47 (1H, d, *J*= 8.6), 7.39 (1H, d, *J*= 8.3), 7.30 (5H, m), 6.86 (1H, m), 6.41 (1H, d, *J*= 9.6), 5.65 (1H, d, *J*= 7.7), 5.09 (2H, s), 4.43 (1H, m), 3.48 (2H, m), 1.69 (4H, m), 1.51 (2H, m).

8-Carbamoyl[*N*-(1-hexadecyl)]-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (37) This compound was prepared from **27** (100 mg, 0.4 mmol) and **16** (193 mg, 0.8 mmol) in a procedure similar to that described for **41**. Column chromatography (ether) afforded **37** (34 mg, 0.07 mmol, 19%) as a white powder, mp 65-67 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.81 (1H, d, *J*= 9.7), 7.76 (1H, s), 7.50 (1H, d, *J*= 8.6), 7.43 (1H, d, *J*= 8.8), 6.63 (1H, bt, *J*= 6.1), 6.43 (1H, d, *J*= 9.5), 3.45 (2H, m), 2.15 (2H, m), 1.25 (26H, bs), 0.88 (3H, t, *J*= 6.7). IR ($CHCl_3$ cast): 3315, 2918, 2850, 1731, 1638 cm^{-1}. HR MS m/z: 453.28780 (M^+) (Calcd. for $C_{28}H_{39}NO_4$: 453.28790).

8-Carbamoyl[*N*-(1-decyl)]-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (38) This compound was prepared from **27** (180 mg, 0.8 mmol) and **15** (160 mg, 1 mmol) in a procedure similar to that described for **41**. Chromatography (ether) afforded **38** (106 mg, 0.3 mmol, 36%) as a brownish white powder, mp 139-143 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.82 (1H, d, *J*= 9.8), 7.76 (1H, d, *J*= 0.9), 7.51 (1H, d, *J*= 8.8), 7.44 (1H, dd, *J*= 0.6, 8.6), 6.61

(1H, t, *J*= 6.3), 6.45 (1H, d, *J*= 9.8), 3.48 (2H, m), 1.65 (2H, m), 1.26 (14H, bs), 0.88 (3H, t, *J*= 6.4). IR ($CHCl_3$ cast): 3330, 2924, 1723, 1620 cm^{-1}. HR MS m/z: 369.19491 (M^+) (Calcd. for $C_{22}H_{27}NO_4$: 369.19400).

8-Carbamoyl[*N*-(1-propyl)]-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (39) This compound was prepared from **27** (100 mg, 0.4 mmol) and **14** (84 mg, 1 mmol, dried over NaOH) in a procedure similar to that described for **41**. Chromatography (ether) afforded **39** (21.7 mg, 0.08 mmol, 19%) as a white powder, mp 203-207 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.82 (1H, d, *J*= 9.5), 7.77 (1H, d, *J*= 0.9), 7.51 (1H, d, *J*= 8.6), 7.43 (1H, dd, *J*= 0.9, 8.6), 6.62 (1H, bs), 6.45 (1H, d, *J*= 9.8), 3.47 (2H, m), 1.69 (2H, hx, *J*= 7.5), 1.03(3H, t, *J*= 7.5). ^{13}C-NMR ($CDCl_3$) δ: 160.2, 157.9, 149.9, 147.2, 143.9, 140.7, 126.1, 115.0, 114.1, 110.3, 108.8, 107.2, 41.3, 23.0, 11.5. IR ($CHCl_3$ cast): 3340, 2925, 2853, 1728, 1651 cm^{-1}. HR MS m/z: 271.08405 (M^+) (Calcd. for $C_{15}H_{13}NO_4$: 271.08447).

8-Carbamoyl[*N*-[10-(1-aminodecyl)]]-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (40) Compound **27** (46 mg, 0.2 mmol), mixed with DCC (41 mg, 0.2 mmol) in dry THF. After 15 min., DMAP (catalytic amount), 1,10-diaminodecane (517 mg, 3 mmol) were added and mixture was stirred for 1 h at 0 ^{0}C and 24 h at room temperature. Then $CHCl_3$ and water (20 ml each) were added and the aqueous phase was washed two more times with $CHCl_3$. The organic phase was washed with HCl (0.5 N) three times and dried. The left over was dissolved in EtOAc to afford **40** (65 mg, 0.17 mmol, 84 %) as a white powder, mp >300 ^{0}C (decomposed). ^{1}H-NMR (CD_3OD) δ: 8.17 (1H, d, *J*= 9.7), 7.80 (1H, d, *J*= 8.5), 7.76 (1H, s), 7.69 (1H, d, *J*= 8.8), 6.52 (1H, d, *J*= 9.7), 2.90 (2H, t, *J*= 7.6), 2.45 (2H, m), 1.63 (2H, t, *J*= 7.6), 1.38 (14H, bs). IR (microscope): 3416, 2921, 1726, 1601 cm^{-1}. FAB MS m/z: 384.8 (M^+), ES MS m/z: 385.1 (M^++1), HR ES MS m/z: 385.21342 (M^+ +1) (Calcd. for $C_{22}H_{29}N_2O_4$: 385.212733).

Compounds 18, 41, 42 11-Aminoundecanoic acid, **17** (200 mg, 1 mmol) was dissolved in absolute EtOH (10 ml), containing H_2SO_4 (catalytic amount), and stirred for 24 h. The mixture was neutralized by a solution of $NaHCO_3$ and extracted with EtOAc (three times, 10 ml each) and dried with Na_2SO_4, which yielded **18**, mp 75-77 ^{0}C, (166

mg, 0.7 mmol, 72%). ^{1}H-NMR ($CDCl_3$) δ: 4.06 (2H, m), 2.61 (2H, m), 2.23 (2H, m), 1.53 (2H, m), 1.39 (2H, m), 1.18 (15H, m). IR ($CHCl_3$ cast): 3330, 2918, 2850, 1733, 1487 cm^{-1}. HR MS m/z: 229.03687 (M^+) (Calcd. for $C_{13}H_{27}NO_2$: 229.03744).Compound **27** (207 mg, 0.9 mmol) mixed with $SOCl_2$ (5 ml) and refluxed at 100 °C for 5 h. After evaporation, **18** (45 mg, 0.2 mmol), DMAP (catalytic amount), and THF (10 ml) were added. The mixture was stirred for 10 h, and evaporated. Chromatography (6% MeOH in $CHCl_3$) afforded **8-carbamoyl[*N*-[11-(ethylundecanoate)]]-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (41)** (72 mg, 0.16 mmol, 82%) as a white powder, mp 123-125 °C. ^{1}H-NMR (CD_3OD) δ: 8.06 (1H, d, *J*= 9.5), 7.71 (1H, s), 7.70 (1H, d, *J*= 8.8), 7.58 (1H, d, *J*= 8.8), 6.44 (1H, d, *J*= 9.5), 4.09 (2H, q, *J*= 7.3), 3.90 (2H, t, *J*= 7.0), 3.41 (2H, t, 7.0), 2.27 (3H, t, *J*= 7.3), 2.02 (2H, m), 1.88 (2H, m), 1.30 (12H, bs). ^{13}C-NMR (CD_3OD) δ: 175.5, 162.0, 160.2, 158.3, 151.3, 150.1, 146.2, 128.0, 118.1, 115.7, 115.3, 110.1, 108.8, 68.3, 61.3, 40.6, 35.1, 30.5, 30.4, 30.3, 30.2, 30.1, 28.0, 24.7, 14.5. IR ($CHCl_3$ cast): 3465, 3057, 2926, 1746, 1731, 1632 cm^{-1}. HR MS m/z: 441.21497 (M^+) (Calcd. for $C_{25}H_{31}NO_6$: 441.21515). Compound **41** (30 mg, 0.07 mmol) was subjected to alkaline hydrolysis with NaOH (4.2 mg, 0.1 mmol) in water (10 ml) for 24 h. After washing the mixture with $CHCl_3$, addition of HCl (0.1 N) afforded **8-carbamoyl[*N*-(11-undecanoic acid)]-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (42)** as a precipitate, which was alternatively centrifuged and washed with water three times to afford **42** (22 mg, 0.05 mmol, 76%) as a white powder, mp decomposed at >300 °C.

8-Carbamoyl[*N*-(*N*,*N*'-dicyclohexylurea)]-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (43) (Williams, *et al.*, 1981) This compound was prepared from **27** (230 mg, 1 mmol) in a similar procedure that explained for **38**, but in the absence of R-NH_2 and at room temperature. After 24 h of stirring, the mixture was filtered and the precipitate chromatographed (hexane: ether, 1:1) to afford **43** (126 mg, 0.29 mmol, 29%) as a white powder, mp 196-198 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.82 (1H, d, *J*= 9.8), 7.70 (1H, d, *J*= 0.9), 7.51 (1H, d, *J*= 8.9), 7.42 (1H, dd, *J*= 0.9, 8.5), 6.44 (1H, d, *J*= 9.5), 6.18 (1H, d, *J*= 7.0), 4.27 (1H, m), 4.06, (1H, m), 1.90 (5H, m), 1.65 (5H, m), 1.36 (5H, m), 1.14 (5H, m). IR ($CHCl_3$ cast): 3311, 2930, 1741, 1708, 1628 cm^{-1}. HR MS m/z: 436.19921 (M^+) (Calcd. for $C_{25}H_{28}N_2O_5$: 436.19983).

1,6-Diethyl hex-2-yne-4-en-1,6-dioate (44) Ethyl propiolate (65 mg, 0.7 mmol), and Cu_2O red (28 mg, 0.2 mmol) were suspended in dry DMF (5 ml) under N_2. The stirring mixture heated at 110 ^{0}C for 24 h. Then the mixture was filtered over 0.4 mm diameter particle size silica gel and washed with EtOAc. The EtOAc solution washed with HCl (1 N), then saturated $NaHCO_3$ solution and finally brine, and dried over Na_2SO_4. Evaporation and chromatography (CH_2Cl_2 gradient to CH_2Cl_2: EtOAc, 99.5: 0.5) afforded **44** (20 mg, 0.1 mmol, 15%) as a yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 6.34 (1H,d, *J*= 11.56), 6.20 (1H,d, *J*= 11.66), 4.27 (4H, m), 1.33 (6H, m). ^{13}C-NMR ($CDCl_3$) δ: 164.8, 153.2, 135.5, 121.6, 87.1, 81.5, 62.5, 61.4, 14.2, 14.1. IR ($CHCl_3$ cast): 2983, 2238, 1718, 1251, 756 cm^{-1}. HR MS m/z: 196.07313 (M^+) (Calcd. for $C_{10}H_{12}O_4$: 196.07356).

1-Phenyl-3-phenoxy 2-propenoate (45) and **N,N´-bis[1-(3-phenoxy 2-propenyl)]N,N´-dicyclohexylurea (46)** Propiolic acid (62 µl, 1 mmol), mixed with DCC (206 mg, 1 mmol), in CH_2Cl_2 containing DMAP (catalytic amount). After 15 min, phenol (94 mg, 1 mmol) was added and the mixture stirred for 24 h at room temperature. The reactants were partitioned between water and $CHCl_3$ and the organic extract was chromatographed (gradient made from hexane:CH_2Cl_2, 95:5, to CH_2Cl_2 100%) to afford **45** (70 mg, 0.3 mmol, 29%) and **46** (60 mg, 0.12 mmol, 12%). **45** was separated as a pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 8.01 (1H, d, *J*= 12.2), 7.42 (5H, m), 7.24 (2H, m), 7.13 (3H, m), 5.75 (1H, d, *J*= 12.2). IR ($CHCl_3$ cast): 3069, 1730, 1649, 1186, 1098 cm^{-1}. FAB MS m/z: 241.0 (M^++1), HR MS m/z: 240.07826 (M^+) (Calcd. for $C_{15}H_{12}O_3$: 240.07864). **46** was separated as a white powder, mp 133-138 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.82 (2H, d, *J*= 11.9), 7.40 (4H, m), 7.21 (2H, m), 7.09 (4H, m), 5.61 (2H, d, *J*= 11.9), 4.14 (2H, tt, *J*= 3.5, 11.9), 1.92 (4H, m), 1.73 (4H, m), 1.26 (12H, m). IR ($CHCl_3$ cast): 3017, 2931, 1708, 1654, 1584, 1231, 756 cm^{-1}. FAB MS m/z: 517.7 (M^++ 1), HR MS m/z: 423.22890 (M^+-C_6H_5O), (Calcd. for $C_{31}H_{36}N_2O_5$: 423.22839).

6-[1-(β-D-(Glucopyranosyloxy)]-7-(3-allyloxy)-2*H*-1-benzopyran-2-one (48) This compound was prepared from **47** (10 g, 27.2 mmol) by a procedure similar to that described for **65**, except that the mixture was heated to 90 ^{0}C for 9 h, then EtOH (50 ml)

and $CHCl_3$ (150 ml) were added and the filtrate affords **48** upon evaporation (6.49 mg, 17.1 mmol, 62%) as a yellowish white powder, mp 165- 167 ^{0}C. ^{1}H-NMR (CD_3OD) δ: 7.76 (1H, d, *J*= 9.3), 7.29 (1H, s), 6.88 (1H, s), 6.15 (1H, d, *J*= 9.3), 6.00 (1H, m), 5.63 (1H, m), 5.39 (1H, dt, *J*= 17, 1.5), 5.22 (1H, ddd, *J*= 1.5, 3.0, 10.7), 4.87 (1H, d, *J*= 7.3), 3.85 (1H, d, *J*=7.3), 3.81 (1H, dd, *J*= 2.2, 11.9), 3.60 (1H, dd, *J*= 5.6, 11.9), 3.37 (4H, m). ^{13}C-NMR (CD_3OD) δ: 163.5, 153.9, 152.0, 145.8, 145.4, 133.8, 118.8, 116.3, 113.9, 113.4, 102.9, 102.8, 78.3, 77.9, 74.9, 71.4, 67.4, 62.6. IR (KBr): 3404, 2928, 1758, 1728, 1282, 1076 cm $^{-1}$. HR MS m/z: 381.11781 (M^++1) (Calcd. for $C_{18}H_{20}O_9$: 381.11856).

6-[1-[β-D-(2,3,4,6-Tetraacetyl glucopyranosyloxy)]]-7-(3-allyloxy)-2*H*-1-benzopyran-2-one (49) Compound **48** (26 mg, 0.07 mmol), was dissolved in pyridine (5 ml), DMAP (20 mg), and acetic anhydride (0.5 ml) were added. After stirring for 10 h at room temperature, the mixture was diluted with ether (50 ml) and washed with HCl (3 N) (three times, 10 ml each), water (three times, 10 each) and brine. The organic layer was dried over Na_2SO_4 and the solvent was removed in vacuo. Chromatography (hexane: EtOAc, 3:2) afforded **49** (28 mg, 0.05 mmol, 78%) as a white powder, mp 147-150 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.51 (1H, d, *J*= 9.8), 7.16 (1H, s), 6.77 (1H, s), 6.22 (1H, d, *J*= 9.8), 5.95 (1H, m), 5.39 (1H, dd, *J*= 1.2, 17.4), 5.28 (1H, dd, *J*= 1.2, 10.5), 5.22 (2H, m), 5.10 (1H, m), 4.91 (1H, dd, *J*= 2.4, 5.4), 4.54 (2H, d, *J*= 5.4), 4.21 (1H, dd, *J*= 5.2, 12.2), 4.11 (1H, dd, *J*= 2.4, 12.2), 3.70 (1H, m), 2.00 (3H, s), 1.99 (3H, s), 1.97 (6H, s). ^{13}C-NMR ($CDCl_3$) δ: 170.4, 170.2, 169.4, 169.3, 160.9, 153.2, 152.0, 143.0, 142.5, 131.8, 118.9, 118.7, 114.0, 111.7, 101.8, 100.5, 72.5, 72.1, 71.1, 69.9, 68.3, 61.8, 20.7 (2C), 20.6, 20.5. IR (KBr): 3480, 2928, 1756, 1740, 1232, 1046 cm $^{-1}$. HR MS m/z: 548.15333 (M^+) (Calcd. for $C_{26}H_{28}O_{13}$: 548.15302).

6-Benzyloxy-7-(2-formylmethoxy)-2*H*-1-benzopyran-2-one (52) Compound **51** (26 mg, 0.08 mmol) was added to a stirring mixture of $NaIO_4$ (600 mg, 2.8 mmol), THF (2 ml), MeOH (0.2 ml), H_2O (2 drops) and OsO_4 (2 drops of 5% solution in *t*-BuOH) at room temperature. After 5 h, water and chloroform (20 ml each) were added and the aqueous phase was extracted two more times with $CHCl_3$. The organic phase was detoxified by $Na_2S_2O_4$. Chromatography (benzene:ether, 3:1) of dried organic phase

afforded **52** (18 mg, 0.06 mmol, 70%) as a white powder, mp 73-75 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 9.80 (1H, s), 7.50 (1H, d, *J*= 9.3), 7.31 (5H, m), 6.90 (1H, s), 6.69 (1H, s), 6.23 (1H, d, *J*= 9.3), 5.11 (2H, s), 4.63 (2H, s). IR (KBr): 2960, 2872, 1730, 1278 cm^{-1}.HR MS m/z: 310.08408 (M^+) (Calcd. for $C_{18}H_{14}O_5$: 310.08414).

6-Benzyloxy-7-[3-(1,2 propandiol)]-2*H*-1-benzopyran-2-one (53) Compound **51** (18 mg, 0.06 mmol), was added to a stirring mixture of OsO_4 (2 drops of 5% solution in *t*-BuOH), THF (2 ml), MeOH (0.2 ml), NMO (N-methylmorpholin oxide) (11 mg, 0.09 mmol) at room temperature. After 5 h, water and $CHCl_3$ were added (20 ml each), and the aqueous phase was extracted two more times with $CHCl_3$. Then the organic phase was detoxified by $Na_2S_2O_4$ and chromatographed (10% MeOH in $CHCl_3$) to afford **53** (18 mg, 0.05 mmol, 90%) as a pale yellow powder, mp 170-172 ^{0}C. ^{1}H-NMR (CD_3OD) δ: 7.70 (1H,d, *J*= 9.7), 7.37 (2H, d, *J*= 6.8), 7.25 (3H, m), 7.06 (1H, s), 6.90 (1H, s), 6.16 (1H, d, *J*= 9.3), 5.06 (2H, s), 4.10 (1H, dd, *J*= 4.2, 9.5), 4.01 (1H, dd, *J*= 5.9, 9.3), 3.96 (1H, m), 3.62 (2H, m). ^{13}C-NMR (CD_3OD) δ: 163.5, 154.3, 151.2, 146.7, 145.5, 137.6, 129.4, 129.1 (2C), 128.9, 128.6 (2C), 113.6, 113.0, 102.1, 72.7, 71.6, 71.1, 63.9. IR (KBr): 3412, 2944, 1706, 1280 cm^{-1}. HR MS m/z: 342.11007 (M^+) (Calcd. for $C_{19}H_{18}O_6$: 342.11035).

6,7-Dihydroxy-8-propyl-2*H*-1-benzopyran-2-one (55) Compound **54** (10 mg, 0.03 mmol) dissolved in methanol (5 ml) and Pd-C (10%) powder (catalytic amount) was added. The air in the container was replaced with H_2 by the help of consecutive vacuuming and refilling with hydrogen. After 30 min. of stirring, the reaction was terminated by letting air in. Then the solvent was removed in vacuo and finally chromatography (10% MeOH in $CHCl_3$) afforded **55** (4 mg, 0.02 mmol, 74%) as a dark yellow powder, mp 200-201 ^{0}C. ^{1}H-NMR (CD_3OD) δ: 7.51 (1H, d, *J*= 9.3), 6.68 (1H, s), 6.12 (1H, d, *J*= 9.3), 2.75 (2H, t, *J*= 7.6), 1.57 (2H, m), 1.19 (3H, t, *J*= 7.3). ^{13}C-NMR (CD_3OD) δ: 165.1, 150.7, 147.1, 132.9, 130.4, 118.4, 112.9, 112.5, 110.6, 69.6, 14.8, 11.9. IR (KBr): 3508, 2932, 1680, 1580, 1300 cm^{-1}. HR MS m/z: 220.07298 (M^+) (Calcd. for $C_{12}H_{12}O_4$: 220.07356).

6-Benzyloxy-7-hydroxy-8-[2-(1,1-dimethoxy)ethane)]-2*H*-1-benzopyran-2-one (58) Compound **57** (3 mg, 0.01 mmol) and dl-10 camphorsulphonic acid (catalytic amount), were added to anhydrous methanol (1 ml). After 2 h of stirring at room temperature, NEt_3 (2 drops) was added to quench the progression of the reaction. Water and $CHCl_3$ (5 ml each) were added and the organic layer was washed with water (total three times, 10 ml each). The organic phase was dried over Na_2SO_4 and the solvent was evaporated. Chromatography (hexane: EtOAc, 1:1) yielded **58** (2 mg, 0.006 mmol, 58%) as a yellow powder, mp 45-48 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.48 (1H, d, *J*= 9.3), 7.33 (5H, m), 7.19 (1H, s), 6.77 (1H, s), 6.17 (1H, d, *J*= 9.3), 5.08 (2H, s), 4.68 (1H, t, *J*= 4.9), 3.36 (6H, s), 3.18 (2H, d, *J*= 4.9). ^{13}C-NMR ($CDCl_3$) δ: 161.3, 149.5, 148.8, 144.0, 143.8, 136.0, 128.8 (2C), 128.4, 127.6 (2C), 113.0, 111.8, 111.3, 108.9, 103.8, 71.7, 53.8 (2C), 27.4. IR (KBr): 3376, 2932, 1724, 1580, 1296 cm $^{-1}$. HR MS m/z: 365.12585 (M^+) (Calcd. for $C_{20}H_{20}O_6$: 365.12598).

6-Hydroxy2*H*-furo[2,3-*h*]-1-benzopyran-2-one (59) $Pd(OH)_2$ (catalytic amount) was added to a solution of **60** (15 mg, 0.05 mmol), in MeOH: EtOAc (2:1), and stirred for 1 h, in an atmosphere of H_2 (ambient pressure). Chromatography (benzene: ether, 3:1) of the mixture afforded **59** (6 mg, 0.03 mmol, 59%) as a yellow powder, mp 220-222 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.67 (1H, d, *J*= 9.3), 7.63 (1H, d, *J*= 2.4), 7.06 (1H, d, *J*= 2.4), 6.76 (1H, s), 6.31 (1H, d, *J*= 9.3). IR ($CHCl_3$ cast): 3315, 1716, 1698 cm $^{-1}$. HR MS m/z: 202.02617 (M^+) (Calcd. for $C_{11}H_6O_4$: 202.02661).

6-Hydroxy-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (59), 6-benzoyloxy-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (60), and **5-benzyl-6-hydroxy-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (61)** Compound **57** (15 mg, 0.05 mmol) and p-toluenesulphonic acid (catalytic amount) were added to benzene (5 ml). The stirring mixture was heated at 70 ^{0}C for 1 h. After this period, $CHCl_3$ and $NaHCO_3$ (1 M) (10 ml each) were added, and chloroform solution was washed two more times with alkaline. The organic phase was evaporated and chromatography (benzene: ether, 3:1) yielded **59** (4 mg, 0.02 mmol, 38%), **60** (3 mg, 0.01 mmol, 22%) and **61** (3 mg, 0.01 mmol, 22%). Compound **60** was separated as a yellow powder, mp 95-97 ^{0}C. ^{1}H-NMR ($CDCl_3$, CD_3OD) δ: 7.65 (1H, d, *J*= 2.4), 7.64

(1H, d, *J*= 8.8), 7.43 (2H, m), 7.33 (3H, m), 7.08 (1H, d, *J*=2.0), 6.77 (1H, s), 6.32 (1H, d, *J*= 9.3), 5.2 (2H, s). IR ($CHCl_3$ cast): 2930, 1724, 1579, 1307 cm^{-1}. HR MS m/z: 292.07348 (M^+) (Calcd. for $C_{18}H_{12}O_4$: 292.07355). **Compound 61** was isolated as a dark yellow powder, mp 214-217 ^{0}C (decomposed). ^{1}H-NMR ($CDCl_3$) δ: 7.87 (1H, d, *J*= 9.8), 7.63 (1H, d, *J*= 2), 7.16 (2H, m), 7.05 (1H, d, *J*=2), 7.01 (3H, m), 6.22 (1H, d, *J*= 9.8), 5.31 (1H, s), 4.31 (2H, s). ^{13}C-NMR ($CDCl_3$) δ: 161.6, 147.0, 145.2, 142.5, 140.1, 137.6, 128.4, 128.4, 127.9, 127.9, 125.9, 123.0, 118.5, 116.2, 115.2, 113.3, 104.5, 30.6. IR (KBr): 3168, 2924, 1696, 1574, 1280 cm^{-1}. HR MS m/z: 292.07276 (M^+) (Calcd. for $C_{18}H_{12}O_4$: 292.07355).

5,6-Dimethoxy-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (63) Compound **59** (5 mg, 0.025 mmol) was added to a mixture of dimethyl sulfate (7.3 mg, 0.06 mmol), K_2CO_3 (35 mg, 0.25 mmol), in MeOH (5 ml), and the mixture was stirred for 5 h at room temperature. The reacting materials were partitioned between water and chloroform (20 ml each), and the organic phase was extracted two more times with $CHCl_3$. Then the organic phase was washed three times with water and chromatographed (hexane:EtOAc, 2:1), to afford **63** (1 mg, 0.004 mmol, 16%) and **62** (2mg, 0.009 mmol, 37%). **63** was obtained as a yellow powder, mp 83-85 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 8.02 (1H, d, *J*= 9.8), 7.59 (1H, d, *J*= 2.5), 7.02 (1H, d, *J*= 2), 6.30 (1H, d, *J*= 9.8), 4.08 (3H, s), 3.97 (3H, s). ^{13}C-NMR ($CDCl_3$) δ: 165.5, 162.8, 158.0, 145.4, 140.5, 139.9, 128.5, 117.0, 114.5, 113.8, 104.3, 62.4, 61.2. IR ($CHCl_3$ cast): 2922, 1731 cm^{-1}. HR MS m/z: 246.05266 (M^+) (Calcd. for $C_{13}H_{10}O_5$: 246.05283).

6-Acetoxy-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (64) This compound was prepared from **59** (4 mg, 0.02 mmol) by a procedure similar to that described for **67**. Yield: **64** (3 mg, 0.01 mmol, 62%) as a yellow powder, mp 134-136 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.68 (1H, d, *J*= 9.3), 7.63 (1H, d, *J*= 2.2), 7.12 (1H, s), 7.10 (1H, d, *J*= 2.2), 6.36 (1H, d, *J*= 9.3), 2.10 (3H, s). ^{13}C-NMR ($CDCl_3$) δ: 168.4, 160.4, 148.8, 146.6, 146.4, 144.3, 132.9, 119.3, 116.2, 115.2, 113.8, 105.1, 20.9. IR (KBr): 1774, 1732, 1306, 1210 cm^{-1}. HR MS m/z: 244.03758 (M^+) (Calcd. for $C_{13}H_8O_5$: 244.03717).

6-(3-Allyloxy)-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (65) Compound **59** (8mg, 0.04 mmol), dissolved in dry DMF (5ml), which contained allyl bromide (2.8 mg, 0.04 mmol), and K_2CO_3 (55.2 mg, 0.4 mmol). After 30 min. of stirring at room temperature, first $CHCl_3$ then water (20 ml each) were added and the aqueous phase was extracted two more times with $CHCl_3$. The organic phase was then washed three times with water and chromatographed (benzene: ether, 3:1) to afford **65** (7 mg, 0.03 mmol, 72%) as a yellow powder, mp 125-127 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.67 (1H, d, *J*= 9.8), 7.64 (1H, d, *J*= 2.4), 7.07 (1H, d, *J*= 2.4), 6.74 (1H, s), 6.33 (1H, d, *J*= 9.8), 6.06 (1H, m), 5.42 (1H, ddd, *J*= 1.5, 3.0, 17.1), 5.29 (1H, ddd, *J*= 1.2, 2.7, 10.7), 4.70 (2H, m). ^{13}C-NMR ($CDCl_3$) δ: 162.5, 153.8, 148.9, 146.0, 144.3, 142.0, 132.4, 118.7, 117.1, 114.5, 113.6, 105.6, 104.6, 70.4. IR (KBr): 1706, 1582, 1344 cm $^{-1}$. HR MS m/z: 242.05703 (M^+) (Calcd. for $C_{14}H_{10}O_4$: 242.05791).

6-(1-Propyloxy)-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (66) 5 mg (0.031 mmol) of **65** was dissolved in 5 ml EtOAc with few drops of MeOH. A catalytic amount of Pd-C (10%) was added and the mixture was hydrogenated at atmospheric pressure. After 30 min., the mixture was filtered. Chromatography (10% MeOH in $CHCl_3$) of the mixture afforded **66** (5 mg, 0.02 mmol, 73%) as a greenish-yellow powder, mp 141-143 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.68 (1H, d, *J*= 9.5), 7.64 (1H, d, *J*= 2.0), 7.06 (1H, d, *J*= 2.0), 6.72 (1H, s), 6.33 (1H, d, *J*= 9.5), 4.09 (2H, t, *J*= 6.6), 1.87 (2H, hex, *J*= 6.9), 1.04 (3H, t, *J*= 7.4). ^{13}C-NMR ($CDCl_3$) δ: 161.1, 147.2, 145.9, 144.4, 143.0, 142.5, 118.6, 114.4, 113.6, 104.8, 104.5, 71.1, 22.5, 10.4. IR (KBr): 2924, 2856, 1726, 1580, 1344 cm $^{-1}$. HR MS m/z: 244.07328 (M^+) (Calcd. for $C_{14}H_{12}O_4$: 244.07356).

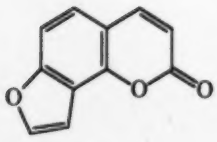
5-Benzyl-6-acetoxy-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (67) A mixture of **61** (2 mg, 0.007 mmol), acetic anhydride (0.5 ml), CH_2Cl_2 (5 ml), DMAP (dimethylaminopyridine) (catalytic amount), and NEt_3 (0.7 mg, 0.007 mmol) was stirred for 30 min at room temperature. The reaction mixture was partitioned between water and $CHCl_3$ (20 ml each), followed by two more extractions with $CHCl_3$. The organic layer then was washed three times with water, HCl (0.1 N) and finally with 0.1 M solution of K_2CO_3. Chromatography (10% MeOH in $CHCl_3$) of the dried organic mixture afforded

67 (2 mg, 0.005 mmol, 78%) as a yellow powder, mp 128-131 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.79 (1H, d, *J*= 9.9), 7.61 (1H, d, *J*= 2.0), 7.18 (2H, m), 7.09 (1H, d, *J*= 2.2), 7.03 (3H, m), 6.25 (1H, d, *J*= 9.9), 4.19 (2H, s), 2.32 (3H, s). ^{13}C-NMR ($CDCl_3$) δ: 175.1, 168.2, 146.0, 145.3, 141.4, 138.8, 128.8 (2C), 127.9 (2C), 126.6, 125.5, 122.9, 117.4, 114.4, 113.0, 105.2, 104.8, 30.9, 20.3. IR (KBr): 1760, 1738, 1204 cm $^{-1}$. HR MS m/z: 344.08455 (M^+) (Calcd. for $C_{20}H_{14}O_5$: 344.08414).

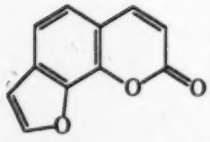
5-Benzyl-6-propargyloxy-2*H*-furo[2,3-*h*]-1-benzopyran-2-one (68) 12.5 µl (0.07 mmol) propiolic acid was mixed with DCC (42 mg, 0.07 mmol) in CH_2Cl_2 (5 ml). After 30 min. **61** (12 mg, 0.04 mmol.) was added. The reaction mixture was filtered after 3 h, and chromatography (CH_2Cl_2) of the filterate afforded **68** (2.1 mg, 0.006 mmol, 15.2%) as a yellow powder, mp 139-142 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 7.94 (1H, d, *J*= 10.3), 7.77 (1H, d, *J*= 2.4), 7.31 (5H, m), 7.24 (1H, d, *J*= 2.0), 6.42 (1H, d, *J*= 10.3), 4.40 (2H, s), 3.2 (1H, s). ^{13}C-NMR ($CDCl_3$) δ: 159.9, 149.6, 148.4, 147.5, 146.3, 141.2, 138.4, 128.9 (2C), 128.0 (2C), 126.8, 125.6, 117.6, 115.0, 114.7, 113.1, 104.9, 82.0, 78.2, 31.4. IR (KBr): 2128, 1738, 1644, 1186 cm $^{-1}$. HR MS m/z: 344.06843 (M^+) (Calcd. for $C_{21}H_{12}O_5$: 344.06848).

Antifungal Susceptibility test: The antifungal activity was measured based on the recommendations of NCCLS (1992). The fractions or the compounds were dissolved in acetone and diluted in a two fold manner in RPMI 1640 (pH=7.0) in 96 microwell plates. The MIC was the minimum concentration of the agent that shows a full inhibition of the fungal growth in the well, examined by naked eyes.

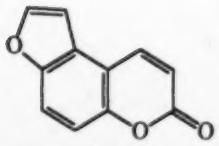
Cell toxicity test: *In vitro* KB cell toxicity was determined for a few compounds, which showed better antifungal activity. The MTT method was used for cell toxicity test, which involves conversion of MTT to blue colored formazan derivative by the active cells, according to the Hansen *et al.* (1989) procedure. The values are average of three separate experiments and expressed as TD_{50}, µg ml^{-1}.



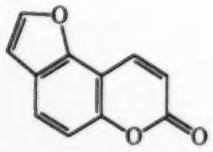
28: Angelicin



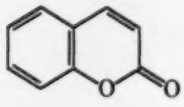
1: Pseudoisopsoralen



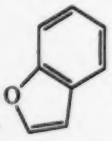
2: Isopseudopsoralen



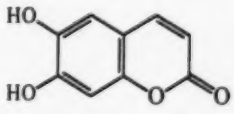
3: Allopsoralen



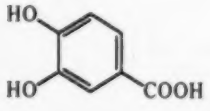
4: Coumarin



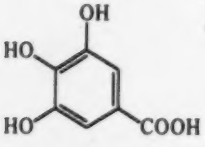
5: Benzofuran



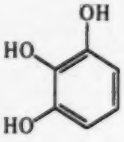
6: Esculetin



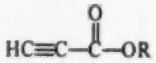
7: 3,4 dihydoxybezoic acid



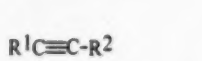
8: Gallic acid



9: Pyrogallol



10: R = H, Propiolic acid
11: R = Et, Ethyl propiolate



12: R^1, R^2 = $Si(Me)_3$
13: R^1 = EtCHOH, R^2 = COOMe

$H_2N(CH_2)_nCH_3$

14: n = 2
15: n = 9
16: n = 15

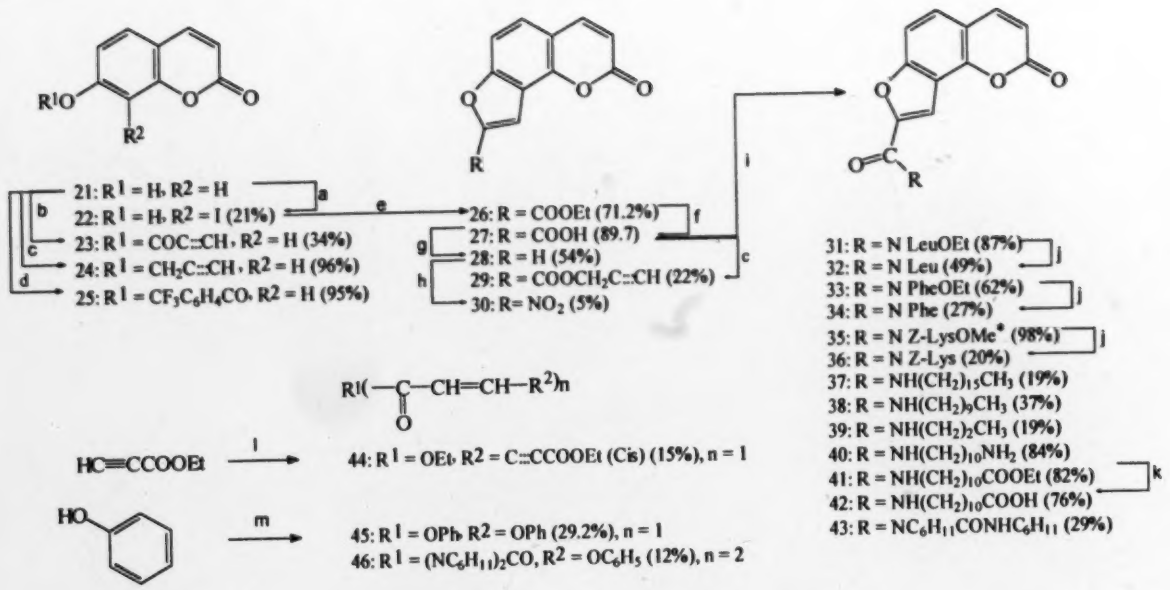
$H_2N(CH_2)_{10}R$

17: R = COOH
18: R = COOEt
19: R = NH_2

$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

20: Spermin

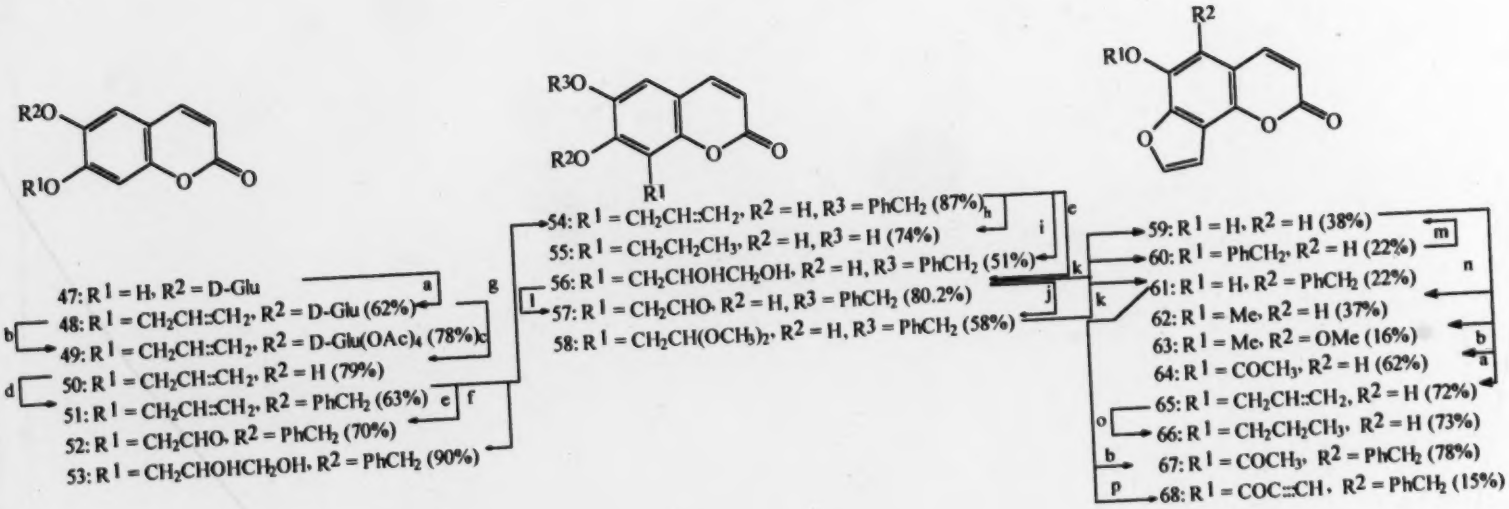
Figure 5-1. Structure of some coumarins and other simple building blocks used in this study.


21: R1 = H, R2 = H
22: R1 = H, R2 = I (21%)
23: R1 = COC≡CH, R2 = H (34%)
24: R1 = CH2C≡CH, R2 = H (96%)
25: R1 = CF3C6H4CO, R2 = H (95%)
26: R = COOEt (71.2%)
27: R = COOH (89.7)
28: R = H (54%)
29: R = COOCH2C≡CH (22%)
30: R= NO2 (5%)
31: R = N LeuOEt (87%)
32: R = N Leu (49%)
33: R = N PheOEt (62%)
34: R = N Phe (27%)
35: R = N Z-LysOMe* (98%)
36: R = N Z-Lys (20%)
37: R = NH(CH2)15CH3 (19%)
38: R = NH(CH2)9CH3 (37%)
39: R = NH(CH2)2CH3 (19%)
40: R = NH(CH2)10NH2 (84%)
41: R = NH(CH2)10COOEt (82%)
42: R = NH(CH2)10COOH (76%)
43: R = NC6H11CONHC6H11 (29%)
HC≡CCOOEt
44: R1 = OEt, R2 = C≡CCOOEt (Cis) (15%), n = 1
45: R1 = OPh, R2 = OPh (29.2%), n = 1
46: R1 = (NC6H11)2CO, R2 = OC6H5 (12%), n = 2

(a) I_2, KI/ H_2O; (b) CH≡CCOOH, DCC/ CH_2Cl_2; (c) CH≡CCH$_2$Br, K_2CO_3/ DMF; (d) $CF_3C_6H_4COCl$, DMAP, NEt_3/ THF; (e) CH≡CCOOEt, Cu_2O/ DMF, 110°C; (f) NaOH/ H_2O;
(g) Cu/ quinoline; (h) HNO_3/ $(Ac)_2O$, -10°C; (i) 31, 33, 35; DCC, NMM, HBT, H_2NR/ THF; (i) 37-41; I. $SOCl_2$, II. H_2NR/ THF, DMAP; (i) 43; DCC, DMAP/ THF;
(j) NaOH (4 eq.)/ H_2O; (k) NaOH (1.5 eq.)/ H_2O; (l) Cu_2O/ DMF, 110°C ; (m) CH≡CCOOH, DCC, DMAP/ CH_2Cl_2

* Z-LyzOMe, is $NH_2(CH_2)_4CH(COOMe)NHCOOCH_2C_6H_5$

Chart 5-1.


47: R1 = H, R2 = D-Glu
48: R1 = CH2CH=CH2, R2 = D-Glu (62%)
49: R1 = CH2CH=CH2, R2 = D-Glu(OAc)4 (78%)
50: R1 = CH2CH=CH2, R2 = H (79%)
51: R1 = CH2CH=CH2, R2 = PhCH2 (63%)
52: R1 = CH2CHO, R2 = PhCH2 (70%)
53: R1 = CH2CHOHCH2OH, R2 = PhCH2 (90%)
54: R1 = CH2CH=CH2, R2 = H, R3 = PhCH2 (87%)
55: R1 = CH2CH2CH3, R2 = H, R3 = H (74%)
56: R1 = CH2CHOHCH2OH, R2 = H, R3 = PhCH2 (51%)
57: R1 = CH2CHO, R2 = H, R3 = PhCH2 (80.2%)
58: R1 = CH2CH(OCH3)2, R2 = H, R3 = PhCH2 (58%)
59: R1 = H, R2 = H (38%)
60: R1 = PhCH2, R2 = H (22%)
61: R1 = H, R2 = PhCH2 (22%)
62: R1 = Me, R2 = H (37%)
63: R1 = Me, R2 = OMe (16%)
64: R1 = COCH3, R2 = H (62%)
65: R1 = CH2CH=CH2, R2 = H (72%)
66: R1 = CH2CH2CH3, R2 = H (73%)
67: R1 = COCH3, R2 = PhCH2 (78%)
68: R1 = COC≡CH, R2 = PhCH2 (15%)

(a) ally bromide, K_2CO_3/ DMF; (b) acetic anhydride, DMAP, NEt_3/ CH_2Cl_2; (c) H_2SO_4/ H_2O; (d) benzyl bromide, K_2CO_3, NaI/ DMF; (e) $NaIO_4$, OsO_4/ THF, MeOH, H_2O; (f) NMO, OsO_4/ THF, MeOH, H_2O; (g) 215-225°C, 1mmHg; (h) H_2, Pd-C/MeOH; (i) NMO, OsO_4/ Acetone, EtOAc, H_2O; (j) 10-camphorsulfonic acid/ MeOH; (k) p-toluenesulfonic acid/ C_6H_6 (total yield 82%); (l) $NaIO_4$/ THF, MeOH, H_2O; (m) H_2, $Pd(OH)_2$/ EtOAc, MeOH; (n) $(CH_3)_2SO_4$, K_2CO_3/ MeOH (total yield 52%); (o) H_2,Pd-C/ EtOAc, MeOH, (p) CH≡CCOOH, DCC/ CH_2Cl_2.

Chart 5-2.

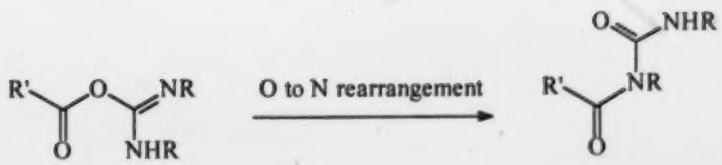
Results

Chemistry

The structures of several known compounds used in this study are shown in Figure 5-1. Synthetic procedures for target compounds are summarized in Charts 1-2. Compounds **22, 26-28** were synthesized according to Zubía *et al.* (1992). To synthesize different coumarins and furocoumarins, umbelliferone and esculin were used as the starting material. Compound **30** was made using the method described earlier (Bastian, *et al.*, 1981). The 8-substituted series of dihydroxycoumarins were prepared through the Claisen rearrangement of compound **51**.

With a few modifications, compounds **59** and **62** were prepared mainly by the procedure mentioned before (Ahluwalia, *et al.*, 1977; Seshadri, *et al.*, 1962). One of the major modifications applied to these sets of reactions was replacement of PPA (polyphosphoric acid) with *p*-toluenesulfonic acid, which gave a better yield and less side reactions. Compound **61**, which is reported for the first time, has an interesting synthetic procedure in our study. The mechanism leading to the production of **61** could be a Fries-type rearrangement of the benzylic group from position 6 to position 5 (Trost, *et al.*, 1991a). Our observation suggests that time of the reaction works in favor of **61** and against **60**, the longer the reaction, the larger the yield of **61**. Compound **63** was a novel product of the reaction "n" in chart 2. The reaction also led to compound **62** as expected. The relative ratio of **62**:**63** was 2:1.

Compounds **46** and **43** are a result of O to N acyl shift of DCC intermediate adducts at room temperature (Trost *et al.*, 1991b; Mikolajczyk, *et al.*, 1981; Williams, *et al.*, 1981). In reaction "m" of Chart 1., none of the products have a triple bond. This indicates that addition of phenol to the triple bond of propiolic acid is a highly favored reaction; and this is in turn followed by either possibilities at the other end of propiolate, esterification step to form **45** or rearrangement of propiolate residue on DCC to give **46**.



N-Acylurea

Bioactivity

The antifungal activity of different coumarin derivatives is shown in Table 5-1. Different angular furocoumarins **1-3** and **28** exhibit similar antifungal spectrum and potency. The activity of individual segments, i.e., coumarin (**4**) and benzofuran (**5**) are much less compared with the original furocoumarins. Other compounds with a phenolic hydroxyl group (**6-9,** and **21**), do not show promising activity either. In this respect, pyrogallol has the strongest effect.

By comparing the antifungal activity of simple alkyne derivatives (**10-13**, and **44**), it is clear that the most potent compound is **44** followed by compound **13**. Alkyne derivatives **11** and **12** do not show any bioactivity and that of **10** is weak. In this group, the mere existence of the triple bond is not enough for a molecule to show antifungal property. However, the activity is limited to molecules with electron withdrawing groups attached to both sides of the triple bond.

Among the alkylamines (**14-16**), and other long chain derivatives (**17-20**), there are several active molecules. In the alkylamine series, hexadecylamine (**16**) exhibits a very strong antifungal activity, especially against *Cryptococcus* and *Saccharomyces*. It is possible to mention that by enlarging the chain length, the activity in this group increases. Addition of one or more polar groups to the alkyl chain makes it a weaker antifungal. This effect is evident for compounds **17-20** compared to**15** and **16**. Ionization might be a negative factor in the final result of bioactivity. The antifungal property of compound **18,** with an esterified carboxyl group, is increased to a minimum of 4-6 fold compared with **17**.

In the 7-mono-substituted coumarins (**21-25**), there is only one interesting compound, which is **23**. The other derivatives are either very weak or inactive on *C. albicans*. Esterification of **21** by a pharmacophoric group such as $CF_3C_6H_4CO$- to produce **25** do not enhance the activity, while a better effect is observed in the case of adding propiolyl group to make **23**. It is of interest that the activity drops by changing the carbonyl group of **23** to methylene in **24**.

The carbonyl group was used to link different moieties to the furanocoumarin nucleus. Although the carboxyl derivative **27** is not considered active, the ethyl ester derivative **26** gained some activity. Addition of alkyne group to make the structure **29** only improved the activity against *Cryptococcus*. In simple furanocoumarin derivatives, **26-30**, compounds **28** and **30** show considerable activity. Compound **30** is a potent antifungal compared to the simple furanocoumarins and carboxyangelicin **27**, however, having its low synthetic yield improved, it could be a precursor for more derivatives and further development.

Among the amino acid derivatives **31-36**, the best effect is found in the phenylalanine-containing molecule with a free carboxyl group (**34**). This group of compounds is totally inactive against *Candida*, very weak to *Cryptococcus*. However, it is interesting that contrary to many other tested compounds in this report, **34** and **36** show moderate activity against *Aspergillus*.

The activity found in **26** is extended to the amide derivative **39** and is improved in **38**, but stopped or reversed in **37**. The addition of either acidic or alkaline polar groups to the side chain of **38**, like in **40** and **42**, reduce the activity again. Derivative **43**, which has bulky non-polar groups together with polar amide groups, is totally inactive. However, compound **46** with similar cyclic lipophilic groups exhibits a weak activity.

Derivative **43**, which carries DCC residue, is totally inactive. Except the phenyl carboxylate derivative **45**, which has a moderate activity against *Cryptococcus*, compounds **45** and **46** are not promising in this regard.

Compounds **50-53** are 6,7 di-substituted coumarins, which exhibit weak or no activity. In the series of 6,7,8 tri-substituted coumarin derivatives, **54-58**, compound **55** has the strongest and broadest spectrum of activity. Compound **55** is also the only compound of this group, which has free 6-OH, that probably contributes to its potency. However, as no other similar compound in this series has been tested, it would not be a proper conclusion to attribute the observed activity to only this functional group.

Replacement of position 8 hydrogen with an alkyl group as in **55** results in an increase in antifungal properties compared to **6**. Alkylation of one or both hydroxyl groups in **6** can affect bioactivity in opposite directions; leaving one hydroxyl free makes a better antifungal. In the case of esculin (**47**), a naturally occurring coumarin glycoside, protecting the phenolic hydroxyl to make **48** does not improve the activity, and so does protection of sugar hydroxyls by the acetyl moiety (**49**).

The more polar derivative **53** is reasonably more potent than **51** and **52**. The 8-substituted derivative **58** is more active than **56**. Furanocoumarins with a substituent on position 6 were also examined. In this group, **64, 67** and **68** show the strongest activity against *Candida* and *Cryptococcus*. Among the compounds with a substituent replacing hydrogen in 6-OH of **59** i.e., **60, 62, 64, 65**, and **66**, the strongest activity against *Cryptococcus* was exhibited by **64**, which is in the range of **59** with the intact 6-OH. A similar comparison among the 5-substituted compounds in this series, **61, 67, 68** indicates that the compounds **67** and **68** with protected 6-OH are stronger antifungals.

The KB cell line toxicity of several compounds is shown in Table 5-2. As can be seen, compound **44** has a strong toxicity, which is followed by **15**. On the other hand, the integration of these moieties with coumarin nucleus has reduced their cytotoxicity to a great extent in **23** and **38**. Amino acid derivatives, **31**, and **33-36** show very weak or no toxicity toward KB cells. Angelicin itself is almost non-toxic to KB cells. Compound **61** with a free hydroxyl group is also non-toxic.

The estimated log *P* and log $D_{7.0}$ values of selected coumarins are shown in Table 5-3. Higher log *P* values are indicative of an increase in hydrophobic character. The log *D*, however, put ionization properties of different functionalities into consideration, so that

the calculated value at pH 7, log $D_{7.0}$, are shown here. It is predictable that the ionization of an acidic or alkaline group at pH 7 reduces the hydrophobicity of the whole molecule, and therefore log $D_{7.0}$ values are decreased, e.g., compare the values of log $D_{7.0}$ for **17** and **18**. Increase in the number of carbons and molecular size usually increases hydrophobicity; for example the longer the hydrocarbon chain in **16** gives more hydrophobic character in this molecule compared with **15** and **14**.

Table 5-1. *In vitro* antifungal activity of the coumarin compounds, expressed as MIC values (μg/ml). Medium, RPMI 1640, inoculum 5×10^4 CFU/ml, temperature 35^0C, incubation period 24-48 h.

Compound no.	*Candida albicans* ATCC 14053	*Cryptococcus neoformans* KF-33	*Saccharomyces cerevisiae* PLM 454	*Aspergillus niger* PLM 1140
1	250	62.5	62.5	62.5
2	250	N/A[a)]	62.5	62.5
3	N/A	N/A	125	62.5
4	N/A	500	1000	-[b)]
5	1000	500	500	-
6	N/A	1000	1000	-
7	N/A	N/A	N/A	-
8	500	250	1000	-
9	250	125	-	-
10	500	250	250	-
11	N/A	N/A	N/A	N/A
12	N/A	N/A	N/A	-
13	62.5	15.6	31.3	-
14	125	62.5	250	N/A
15	<7.8	<7.8	<7.8	15.6
16	15.6	<1.9	<1.9	7.8
17	500	250	500	500
18	62.5	15.6	7.8	62.5
19	500	125	250	250
20	250	125	125	125
21	1000	500	-	500
22	1000	500	-	1000
23	15.6	62.5	125	250
24	N/A	500	125	N/A
25	N/A	500	500	-
26	1000	250	-	1000
27	N/A	2000	-	2000
28	62.5	250	125	62.5
29	N/A	62.5	N/A	N/A

30	15.6	3.9	<1.9	15.6
31	N/A	1000	-	N/A
32	N/A	1000	-	2000
33	N/A	2000	125	1000
34	N/A	500	250	125
35	N/A	N/A	N/A	1000
36	N/A	N/A	500	125
37	N/A	31.3	125	250
38	250	31.3	62.5	125
39	250	125	62.5	N/A
40	250	250	500	500
41	N/A	125	N/A	N/A
42	500	N/A	250	N/A
43	N/A	N/A	N/A	N/A
44	<15.6	<15.6	<15.6	<15.6
45	N/A	125	250	N/A
46	250	500	N/A	500
47	N/A	N/A	N/A	-
48	N/A	N/A	N/A	-
49	N/A	N/A	N/A	-
50	N/A	250	250	-
51	N/A	N/A	N/A	-
52	1000	500	-	-
53	N/A	250	250	-
54	N/A	500	1000	-
55	250	125	62.5	-
56	N/A	1000	1000	-
58	1000	125	250	-
59	250	125	250	-
60	500	250	500	-
61	500	125	500	-
62	500	250	-	-
63	N/A	500	500	-
64	250	62.5	250	-
65	500	500	250	-
66	N/A	250	250	-
67	62.5	500	125	-
68	250	15.6	125	-
AmB	1.6	1.6	0.8	5
Itraconazole	51.2	0.8	0.8	-
Fluconazole	12.8	25.6	6.4	10.0
DMSO	10% v/v	5% v/v	10% v/v	10% v/v
Methanol	>10% v/v	>10% v/v	>10% v/v	>10% v/v
Acetone	>10% v/v	>10% v/v	>10% v/v	>10% v/v

a) N/A, showing no activity up to the maximum concentration that was tested. b) "-", not tested

Table 5-2. *In vitro* cytotoxicity of selected coumarin compounds on the KB cell line, expressed as TD_{50} (μM).

Compound no.	TD_{50}
15	35.0
23	385.5
28	487.3
31	90.7
33	124.5
34	>265.3
36	>203.3
35	108.7
38	139.6
44	3.0
61	282.4
Adriamycin	0.018

Table 5-3. Calculated Log *P* and log $D_{7.0}$ values for selected coumarin compounds.

Compound no.	Log *P*	Log $D_{7.0}$
4	1.75	1.75
5	2.60	2.60
6	0.75	0.74
10	-0.09	-2.94
11	0.82	0.82
13	0.74	0.74
14	0.35	-2.32
15	3.92	1.05
16	6.98	3.70
17	2.82	-1.00
18	3.72	0.88
19	2.33	-2.67
20	-1.03	-10.35
21	1.19	1.18
23	1.55	1.55
26	2.86	2.86
27	1.95	-1.19
28	2.41	2.41
30	2.36	2.36
31	3.61	3.61
32	2.71	-0.37
33	3.90	3.90
34	2.99	-0.26
35	3.68	3.68
36	3.24	1.15
37	9.01	9.01
38	5.95	5.95

39	2.38	2.38
40	4.36	1.43
41	5.75	5.75
42	4.84	2.76
43	5.07	5.07
44(trans)	1.96	1.96
51	3.98	3.98
52	2.74	2.74
53	1.83	1.83
54	3.90	3.90
56	1.59	1.59
58	3.16	3.16
59	1.84	1.06
60	3.95	3.95
61	3.87	3.36
62	2.35	2.35
63	2.21	2.21
64	1.93	1.93
65	2.96	2.96
66	3.32	3.32
67	3.96	3.96
68	4.24	4.24

Discussion

Coumarins have a variety of bioactivities including anticoagulant, estrogenic, dermal photosensitizing activity, antimicrobial, vasodilator, molluscacidal, anthelmintic, sedative and hypnotic, analgesic and hypothermic activity (Soine, 1964). These compounds may also be considered as a defense tool for plants against fungi (Scheel, 1972).

Coumarin inhibits the germination of spores of *Aspergillus niger, Penicillium glaucum,* and *Rhizopus nigricans* (Knypl, 1963). Novobiocin and other 4-hydroxycoumarins are generally ineffective against fungi (Jurd *et al.*, 1970).

To determine the best skeleton for further modification, the antifungal activity of angelicin was compared with **1-3**. As it is shown in Table 5-1., there is not very much difference among these compounds in terms of MIC and spectrum of activity. Angelicin (**28**) and **1**, however, show activity against *Cryptococcus*, and since angelicin is more active on *Candida* than **1**, it was selected for further modifications.

Antifungal activity of 7- substituted coumarin derivatives The methyl ether of 7-hydroxycoumarin has shown activity against *Aspergillus glaucus* and *A. flavus* in both diffuse light and in complete darkness (Jurd, *et al.*, 1970). In a study by Dini *et al.*(1992), several coumarins were isolated and purified from *Cyperus incompletus*. The antifungal tests showed that an aromatic hydroxyl group and/or an extra oxygenated functional group (ether or ester) in the 6 and 7 positions of coumarin were necessary for activity. Alkylated derivatives of 7-hydroxycoumarin may show both antifungal and antibacterial properties. Free hydroxyl group at position 7 of the coumarin nucleus is important for antibacterial activity. It has been suggested that the antifungal activity of furocoumarins may be accounted for by protection of the phenolic OH at position 7 by the etheric bond (Jurd, *et al.*, 1970). In fact, comparison of activities of **21** and those of the 7-OH protected derivatives, i.e., **23, 24, 25** is not fully supportive of this effect.

Antifungal activity of 6, 7- di-substituted coumarin derivatives Comparing the activities of **50** with **51**, and **54** with **55**, reveals that the compounds with free 6-OH have a better activity. However, the same thing is not true for 7-OH, as it is understood from the lack of activity in both **47** and **48**. In addition, the MIC values of **21** and **23-25** are supportive of this idea. It seems that as far as the antifungal activity is concerned, 6-OH is better to be free, while protected 7-OH may or may not (Jurd, *et al.*, 1970) provide more bioactivity.

Antifungal activity of 6,7,8- tri-substituted coumarin derivatives Substitution at position 8 of esculetin (**6**), which resulted in compound **55**, has increased the antifungal activity tremendously. Protection of 6-OH to yield **54** is reduces activity as it happened in the 6,7-disubstituted coumarins. Replacing position 8 with more polar substituent like in **56** diminishes the activity, while transforming the 8-substituent to a more non-polar group like in **58** (compare the log *D* values), regenerates the activity to a great extent.

Antifungal activity of amino acid derivatives of furanocoumarins Amino acid derivatives of cinnamic acid, which is biosynthetically related to coumarins, have been synthesized that exhibit antifungal activity (El-Sayed, *et al.*, 1992). Their activity has been attributed to a better transport ability into the fungal cell. It has been discussed that

an oligopeptide transporter system present in fungal cells helps the transfer of molecules into the cell. The phenomenon of facilitated transport of compounds connected to amino acids into the fungal cell has been described for *Bacillus subtilis* antifungal toxins as well (Rapp, 1988). In addition, a separate report examines the antifungal activity of *N*-coumarin-sulphonylamino acid derivatives (El-Naggar, *et al.*, 1987). Therefore, it was decided that the amino acid derivatives of angelicin be synthesized and tested for their antifungal activity. Computer modeling superimposition of pseudolaric acid, a strong naturally occurring antifungal terpenoid lactone (Li, *et al.*, 1995), and angelicin, proposed position 6 and 2' of angelicin nucleus to be the best locations for the substitution. Efficient synthesis of the 2'-derivative (**27**), encouraged us to continue utilizing this derivative for further works. Although it could be possible to link amino acids through their carboxyl group to an amine moiety at position 2' of angelicin, the low yield of nitro-derivative **30**, leaves this opportunity open for future studies. Three representative amino acids, Leu, Phe, and Lys were chosen and reacted with the carboxylic group of **27**. The best activity in this group was observed for Phe carrying molecule **34**. Although earlier works (El-Naggar, *et al.*, 1987) suggested that an unprotected Leu derivative could be the strongest compound in the coumarin series, the furanocoumarins containing amino acids had a preferred activity with a Phe connected to them. The general MIC values of this group are not considered strong, however they show improvement compared to compound **27.** This superior activity might be due to simple transformation of carboxyl group of **27** to a more non-polar moiety. Comparison of the log *D* values suggests that the generalization in this regard is not sensible. As the calculated lipophilicity estimates of **31**, **33** and **35** are more than that of **26** at pH=7, their activity is not more in all cases. In the same manner, the more polar derivatives **32**, **34**, and **36** do not exhibit an improved antifungal activity in all cases. On the other hand, the compounds containing free carboxyl group with higher log *D* values than that of **27**, like **32, 34,** and **36** indicate a stronger activity compared to **27**. It seems that the amino acid group or at least its carboxyl moiety plays a role in the process of inhibiting fungal cell growth.

Antifungal activity of furanocoumarins carrying a long chain hydrocarbon group at the 2 'position In the homologous series in which simple long chain hydrocarbons are connected to the furanocoumarin skeleton of angelicin, the activities are much better than

the amino acid derivatives. The long chain alkylamines **15** and **16** themselves are potent antifungals. Comparison of their activity with that of spermine (**20**) with four amines per molecule or 1,10-diaminodecane (**19**) with two amines, rules out the possibility that their activity is because of the amine group or a change in the pH of the micro-environment around or inside the fungal cell. By looking at the log *D* values of **14-16**, **19** and **20** in Table 5-3., it becomes clear that the antifungal activities correlate relatively well with the lipophilicity of these molecules.

Substituting one of the amino- groups in **19** with carboxyl- to make **17** does not improve activity. However, the protected derivative **18**, which has ethoxycarbonyl group, shows a much better antifungal profile than **17** and **19**. Compound **17** is present as the zwiterion in the medium solution, and this may hinder its absorption into and distribution within the fungal cell.

The derivatives with these amines, **14-19**, connected to the furocoumarin skeleton, still indicates a correlation between lipophilicity and antifungal activity, compare the log *D* and MIC values of **37-39**. There is a maximum bioactivity at the optimum log *D* value of about 5. It should be noted that in the series with similar chain length i.e., **38**, **40-42**, log *D* alone is not representing the activity. For example, **38** and **41** have very close log *D* value, but their activity is very different from each other. Although lipophilicity plays an important role, factors like electronic effects and polarity seem to be important as well. In the series of molecules, **40-42**, the best activity belongs to **40**. This might be due to the presence of the amino group itself, which can be contributed to the activity of antifungal drugs (Baginski, *et al.*, 1994).

Antifungal activity of 6-substituted furanocoumarins In the furocoumarin series, a simple comparison among compounds **59**, **60**, **62**, **65**, and **66** indicates that the free hydroxyl group in the position 6 is important for antifungal activity. Different alkyl and aryl groups attached to the oxygen at location 6 reduce the activity to almost the same extent. Acetylation of the 6-hydroxyl caused retention of activity in **64** compared to **59**. Acetylation of **61** to yield **67** and **68** generally improved the activity. Although the

addition of the alkynic pharmacophore in **68** improved the activity against *Cryptococcus* dramatically, it had an opposite effect on *Candida* in comparison to **67**.

Addition of a methoxyl group to position 5 imposed a negative effect on the bioactivity in **63** compared to **62**. Providing position 5 with a benzyl moiety decreased the activity. As it is clear from table 5-3., a straight correlation between lipophilicity as represented by log *D* value and antifungal activity cannot be drawn in this group. For example, **59** and **64** have the smallest log *D* values and **60, 67**, and **68** have the largest values in this group. However, the best antifungal activities are found in both groups with high or low log *D* values. The generalization to these observations could be that protection of 6-OH by groups that change the electronic contribution of oxygen 6 on the ring or changing polarity of the functional groups to a favored pattern has an improving effect on the antifungal activity of this group.

Cytotoxicity Safety of the coumarins is another issue to be considered for further development as a drug. Angular furocoumarins like angelicin are less likely to form adducts with DNA during the photoreaction, since they are monofunctional. However, bifunctional coumarins like psoralen form interstrand crosslinks with DNA (Bordin, *et al.*, 1991). A dose of bout 6 times higher is needed to induce 50% cytoplasmic "petit" mutations in the presence of angelicin than in the presence of psoralen (Averbeck, *et al.*, 1975). In general, introduction of the methyl group increases affinity towards DNA, and addition of methyl to positions 5 and 3' seems to be the most effective in this regard (Bordin, *et al.*, 1991). Our cytotoxicity studies indicated that angelicin, **23, 38** and **61** are almost non-toxic and can be considered for further development in this regard. Alkylamines like **15**, which show strong antifungal activity, are cytotoxic as well. A similar case is true for compound **44.**

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Chapter 6
Antifungal Mechanism of Action of Coumarins

Introduction

Curiosity and achieving scientific recognition might be the primary motivations in studying the mechanism of action of new bioactive agents. However, this is not the answer to the high cost and the time consumed by such studies. In fact, there is more than curiosity in this type of research.

Although mode of action studies are more on the basic side of pharmaceutical research, the results can easily find their way into applied research, and drug development. Research on the mechanism of action of antifungal agents can be useful in elucidation of the biological and biochemical processes. For example, sterol biosynthetic inhibitors were useful in the clarification and study of sterol biosynthetic pathways (Sisler, 1996). Mode of action studies can also play a role in finding new targets involved or improving the knowledge of existing targets. In addition to the benefits of this type of study in drug development, it is possible to predict the human toxicity of these agents and refine them. As an example, knowing the action site of azole fungicides, cytochrome P450, any side effect involving human P450 could be possible for this group of antifungals. It is known that ketoconazole is a potent inhibitor of testosterone synthesis and even may be a good candidate for androgen-dependent prostate carcinoma (Vanden Bossche *et al.*, 1992).

In the previous chapter, the synthesis and antifungal activity of several coumarin derivatives was described. Here, we describe studies on the mechanism of action of this group of compounds at the cellular level.

Materials and Methods

***Interaction studies*:**
A modified method of the broth dilution technique that was explained in chapter 2 and 3 was utilized.
Fungal strains: *Candida albicans* ATCC 14053, *Saccharomyces cerevisiae* PLM 454, and *Cryptococcus neoformans* KF-33 were used.

Medium: RPMI 1640 Sigma culture medium with no extra buffer at pH 7, unless otherwise specified. One of the buffers used is MOPS (3-[N-Morpholino]propane-sulfonic acid), 34.5 g/L plus $NaHCO_3$, 2 g/L .

Inoculum: 5×10^3 (CFU/ml) or 5×10^2 (CFU/well) unless otherwise specified. Temperature 30° C, unless otherwise specified.

Checker board method for testing antibiotics interaction The final concentration of each antimicrobial agent diluted in RPMI 1640, ranged from zero to four MIC. Each microwell contained 200 µl of varying concentrations of antimicrobial agent A and varying concentrations of antimicrobial agent B in RPMI medium containing *C. albicans* or other fungal organisms. Control included a growth control that contained medium and fungi without antimicrobial agents and a negative control that contained medium and antimicrobial agents without fungi. All wells were incubated at 35° C for 48 h in duplicate (Lorian, 1986).

Identifying the type of the interaction Based on inhibitory concentration of each agent in the mixture, the decision was made to categorize the interaction as, synergism, antagonism, additive, or subtractive effect. The concentration range of each antimicrobial agent in combination ranged from 1/32 times the MIC to 4 times the MIC. Dilutions of drugs A and B were made with a multichannel micropipet. FIC, Fractional Inhibitory Concentration, is used to judge the interaction type. If "A" would be the test substance, FIC of A= MIC_A in combination / MIC_A alone. FIC index = $FIC_A + FIC_B$. *Synergy*: FIC index ≤ 0.5. *Antagonism*: FIC index ≥ 4. *Interference*: FIC index 0.5< value < 4. (White, *et al.*, 1996). Usually, in each set of interaction studies, concentrations of 1/32 to 4 times the MIC of the antifungal agent were used.

Staining method:

Cells were stained with a 0.5% solution of methylene blue before each examination with light microscope.

***Sterol analysis and extraction*:**

Chemicals: Ergosterol was purchased from Sigma. Squalene, lanosterol/dihydrolanosterol (a mixture of 59.3% and 40% respectively) were purchased from Nakalai (Japan).

Fungal species and growth condition: *C. albicans* ATCC 14053 was cultured on SDA slants for 24 h at 25° C. After this period, fungus was suspended in saline solution to a concentration of 75-77% T at 530 nm. The test compounds were dissolved in water or acetone and added to the medium, RPMI 1640, adjusted to pH = 7.0. The final fungal suspension is equal to 5×10^3 CFU/ml. The standing cultures were kept at 30° C for 21 h.

Sterol extraction: The fungal cells were harvested by centrifugation at 2600 rpm at 0° C for 20 min. They were then washed three times with chilled distilled water and recollected by centrifugation. The cells were divided into two equal sets, one for dry weight measurement and the other for sterol analysis. The first set of cells was dried at 60° C. The second set of cells was suspended in methanol (4 ml) and n-hexane (2 ml). The mixture was sonicated for 1 h. The extraction was repeated twice with a fresh hexane layer each time. Hexane fractions were collected and evaporated in a centrifugal concentrator (Quail, *et al.*, 1993; Sadamori, 1987).

Trimethlylsilyl derivatization of the remaining sterols performed in the presence of BSA (30 µl) [*N*', *O*'-bis-(trimethylsilyl)trifluroacetamide] at 60° C for 1h (Quail, *et al.*, 1993).

To determine the recovery rate, the following method was used: A known amount (A) of ergosterol was added to *Candida* extraction mixture, and every extraction step was followed as test groups. After GC analysis, the average of ergosterol obtained from control groups (C) subtracted from the recovered amount of the groups with added ergosterol (B) and divided by the amount of ergosterol added. The result was multiplied by 100 to express the outcome as percent of recovery.

$$Recovery = (B - C / A) \times 100$$

The recovery of ergosterol obtained by this method was 104.25%.

Analytical method: Sterols were analyzed as their TMS derivatives by GC through a 3% OV-1 capillary column (30 m × 0.25 mm i.d., 0.1 µm film thickness) (Loeffler, *et al.*, 1990). The column was attached to a FID and C-R5A integrator. The carrier gas was He at a flow rate of 40 ml/min. Analysis was carried out under a programmed temperature condition at 170° C (10 min), increased by 10°/min to 230° C (kept for 2 min.), then

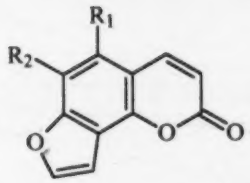
increased by 2°/min to 280° C (and kept for 10 min.). Injector and detector temperatures were set at 290° C and 300° C, respectively. An injection volume of 1 µl was used with a 1:100 split ratio. Cholesterol used as an internal standard (Parks, 1978). GC-MS was performed on a Hewlett-Packard-Jeol JMS-AM 150 system. The capillary column was DB-1 (30 m × 0.25 mm i.d., 0.1 µm film thickness). Conditions were as follows: programmed oven temperature the same as GC condition; He carrier gas at 100 PA; transfer line temperature equal to 250° C; ion source temperature equal to 250° C; electron multiplier at –0.7 kV. The mass range analyzed was 50-70 amu, at one scan per 500 msec. Statistical calculations were done according to Skoog, *et al.* (1990).

Assay method for cyclic AMP phosphodiesterase:

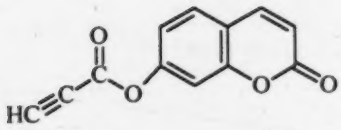
Samples were dissolved in DMSO and tested for their activity against phosphodiesterase of beef heart (Sigma) according to (Nikaido, *et al.*, 1981). The standard reaction mixture (500 µl) containes Tris-HCl (pH 7.5; 0.05 M), $MgCl_2$ (1mM), bovine serum albumin (250 µg), [^{3}H]-cyclic AMP (0.01 mM; 1.2×10^6 dpm), beef heart phosphodiesterase (2.25 mU) and the sample (12.5-100 µg). The reaction was initiated by the addition of [^{3}H]-cyclic AMP. The reaction mixture was incubated for 30 min at 37° C and was stopped by immersing the test tube in boiling water for 3 min. Snake venom nucleotidase (500 µg) was added to the cooled reaction mixture and incubated for 30 min at 37° C. A 50% suspension of Dowex Agl-X8 resin was then added to the reaction mixture. The resin, which absorbed unchanged [^{3}H]-cyclic AMP, was precipitated by centrifugation and the radioactivity of an aliquot of the supernatant containing [^{3}H]-adenosine, resulted in the assay reaction, was measured with liquid scintillation counter. All assays were performed in duplicate. IC_{50} value is the concentration of compound required to give 50% inhibition of phosphodiesterase activity.

Compounds:

Coumarin compounds: Chemicals used in this study as antifungal (except the drugs) were of coumarin types synthesized according to the procedure described in chapter 5. The following structures were used most often.

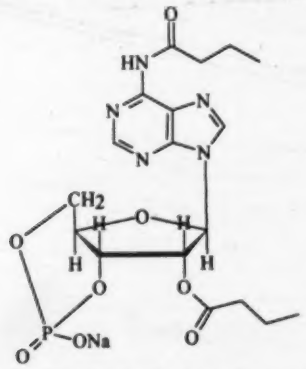


(28) R_1= H, R_2= H, angelicin
(61) R_1= CH_2Ph, R_2= OH

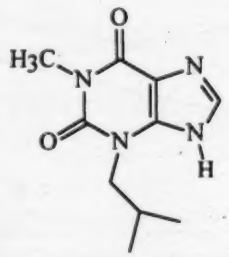


Compound 23

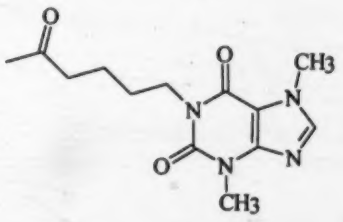
Reference compounds: A group of other drugs and chemicals were used as cAMP analogs, db-cAMP (ICN®), or phosphodiesterase inhibitors, including theophylline, IBMX, pentoxyfylline (all from Sigma®). In addition, the effect of morpholine compounds such as MOPS, NMO, MES on the antifungal activity of coumarins was assessed. The test compounds were dissolved in the following solvents: AmB, itraconazole, ketoconazole, sodium orthovanadate (Na_3VO_4) in DMSO; coumarins, pentoxifylline, IBMX and MM in methanol (depending on the concentration, angelicin might need acetone as co-solvent); fluconazole, db-cAMP, theophylline, hygromycin B, ouabain, MOPS and MES in water; valinomycin in acetone.



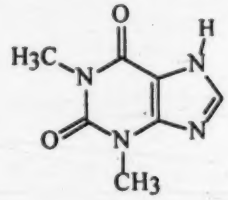
db-cAMP (Bucladesine) sodium salt



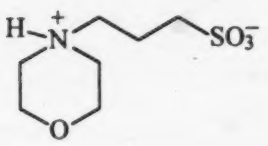
IBMX (3-Isobutyl-1-methylxanthin)



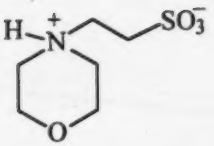
Pentoxifylline



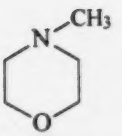
Theophylline



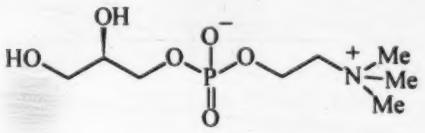
MOPS
(Morpholinopropylsulfonic acid)



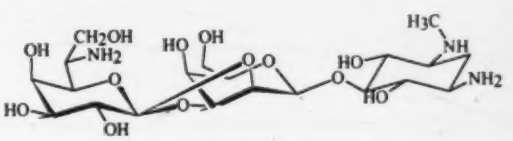
MES
(Morpholinoethan sulfonic acid)



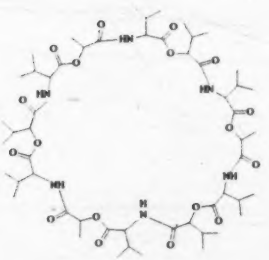
MM
(*N*-Methylmorpholine)



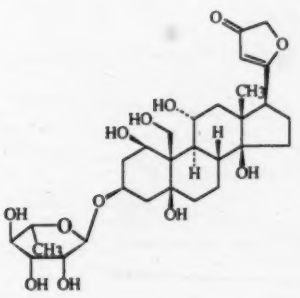
Glycerol phosphorylcholine



Hygromycin B



Valinomycin



Ouabain

Ergosterol binding assay:

To estimate the quality of possible interaction between coumarins and ergosterol, a spectrophotometric method described originally for AmB was used (Mazerki, *et al.*, 1995; Breivik, *et al.*, 1957). AmB (Sigma) and coumarins were dissolved in DMSO and methanol, respectively, at a concentration of 10^{-3} M for AmB, and 10^{-2} for compounds 28 and 23. Ergosterol and cholesterol (Sigma) stock solutions were prepared in $CHCl_3$ at a concentration of 10^{-2} M and stored for a few days in a refrigerator. Sterol working solutions were prepared daily by mixing one part of stock solution with 9 parts of ethanol. Desired amounts of antibiotic and sterol solutions were added to required volumes of distilled water. Spectrophotometric measurements were performed 1 h after mixing. After this period, no changes in the spectra were observed in the control groups. In all experiments, the concentrations of AmB, angelicin, and compound 23 were 1.8, 12.5 and 6.25 μg/ml. The free form of AmB has an absorption band at 409 nm, which does not exist in the spectrum of the complex form, and has a high absorption coefficient. The measurements for angelicin and compound 23 were done at their proper λ_{max} 302 and 317 nm respectively, which did not interfere with sterols absorption region. The results are the average of three separate experiments.

Ion toxicity test:

To evaluate the degree of cell membrane permeability as a result of antifungal agents, fungal cell toxicity in the presence of different concentrations of sodium and calcium solutions was determined (Hemmi, *et al.*, 1995; Taguchi, *et al.*, 1994). Ca^{2+} and Na^{+} added to the medium as $CaCl_2$ and NaCl. Solutions of $CaCl_2$ and NaCl were sterilized separately and added to the medium at room temperature. Angelicin, compound 23, and Amphotericin B were used in the first well concentration of equal to 250, 125, 70 (μg/ml), respectively. To read the results of ranges of inhibition due to higher concentrations of cations, medium observation is necessary to be done by microscope (especially for Ca^{2+} containing wells), as precipitation hinders naked-eye observation. In this experiment, an Olympus CK2 inverted microscope with a magnification of 40 was used.

PH estimation using NMR:

Chemicals: Growth medium RPMI; contained 0.8 g/L sodium phosphate dibasic, and fortified by the addition of potassium phosphate to a final concentration of 15 mM. Glycerol phosphorylcholine was obtained from Sigma and used for calibration purpose (Gillies, *et al.*, 1981).

Preparation of cell suspension for NMR: For studies involving glucose, cells were grown to mid-log phase in RPMI (with 15 mM K_3PO_4) medium in plates as a steady culture at 25°C for 20 h. Likewise, SDA was used to pre-grow cells. Cultures were then cooled on ice to 4° C with gentle agitation, and cells were harvested by centrifugation and washed twice with fresh RPMI (+Pi) medium (Lohmeier-Vogel, *et al.*, 1995). Finally, the cells were suspended in RPMI (+Pi) containing D_2O (20%). At the times 20, and 40 min, the solutions of methanol (to make 15% v/v in the cell suspension) or compound 28 and 23 in methanol (770 and 57 μg/ml cell suspension respectively), and glucose (10.1 mg) were added to the NMR tube, respectively.

^{31}P-NMR spectroscopy: NMR spectra were obtained at 202.3 MHz using a Varian Unity-500 MHz spectrometer operating in the Fourier-transform mode. Each spectrum was acquired with 45° pulses at a repetition rate of 1.60 s and 1050-10,000 scans were accumulated. Peak position of inorganic phosphate dissolved in the culture medium used as an internal reference, while the samples containing the test solutions were adjusted and plotted according to this reference. Temperature was ambient throughout the measurements. Cells were transferred to 5-mm NMR tubes and made to a density of about 2×10^9 cells/ml in RPMI (with 15 mM K_3PO_4 added) medium with final concentration of 20% (v/v) D_2O. D-Glucose was added to the medium in a concentration of 86 mM in the specified groups. The inorganic phosphate was used for calibration of the chemical shifts of orthophosphates for the estimation of changes in intra- and extracellular pH.

Electron Microscopy:

Preparation of the cells for electron microscopy: *C. albicans* cells, cultured on SDA plates, were used to make an inoculum of 10^6 (CFU/ml) in saline, equal to a transmittance of 75-77% at 530 nm, and transferred to tubes containing RPMI 1640 medium to make a final cell count of 5×10^3 (CFU/ml) in the tubes. The antifungal agents were added at subinhibitory concentrations to allow the cells to grow and make enough cells for the microscopic examination. The final concentrations of fluconazole, AmB, angelicin, compounds 61, and 23 in the medium were 1, 1, 42, 20, and 2.5 μg/ml, respectively. Cells were incubated at 25°C. To the control groups equal volume of solvent used for antifungal agents, methanol (33 μl), was added.

SEM (Scanning Electron Microscopy): After appropriate incubation and treatment regiments, the cultures were centrifuged and pelleted cells washed with isotonic phosphate buffer (pH 7), fixed in 2.5% glutaraldehyde at 4° C for 12 h, and washed again in 0.214 (M) Millonig's phosphate buffer (pH 7.4). They were then postfixed with 2% osmium tetroxide for 2 h. The cells were dropped onto a poly-L-lysin coated coverslip (12 mm in diameter) and stayed for 10 min, and dehydrated in an increasing series of ethanol solutions. The samples were put in a critical point drier, and coated with gold in argon atmosphere using an Edwards Sputter Coater S150B (England). The specimens were examined with a Hitachi S-2500 scanning electron microscope (Tokyo, Japan).

TEM (Transmission Electron Microscopy): Aliquots of the centrifuged pellets (10 min, 10 000 rpm) of *C. albicans,* cultured as described above, were prefixed in a mixture of 2.5% (v/v) glutaraldehyde-0.2 M Millonig's phosphate buffer, pH 7.4, then postfixd with 2% (w/v) osmium tetroxide for 3 h at room temperature. In the specified cases, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 6% tannic acid (pH was adjusted to pH 7.4 by NaOH) was used for the purpose of fixation and staining. Samples were dehydrated in ethanol series and embedded in Spurrs resin. Specimens were cut on an ultramicrotome as 0.1 μm thickness and collected on 200-mesh copper grids with a formvar supporting film (Sigma). Thin sections were stained with uranyl acetate and lead citrate. Sections were examined by a Hitachi H-7000 transmission electron microscope (Tokyo, Japan) operated at 75 kV.

Results

Effect on ergosterol and ergosterol-related targets: One of the early objectives of this study is to determine the similarity of action between the coumarin antifungals and the antifungal drugs that are on the market. For this purpose, the interaction studies carried out show that there is an antagonistic relationship between ketoconazole and compound 23 (Fig. 6-1). This interaction has an FIC value of 250 for *C. neoformans* and 3.9 for *S. cerevisiae*. In both cases, the antagonism is evident.

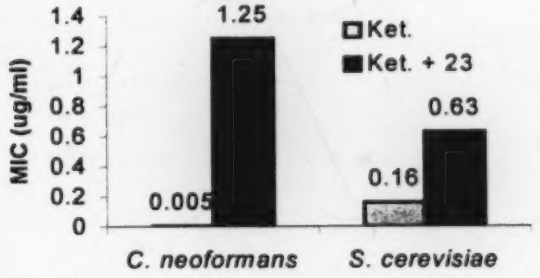


Figure 6-1. Interaction between ketoconazole and compound 23. Medium, RPMI 1640, with MOPS buffer, inoculum 5×10^3 (CFU/ml), temperature 37°C, incubation period 48 h.

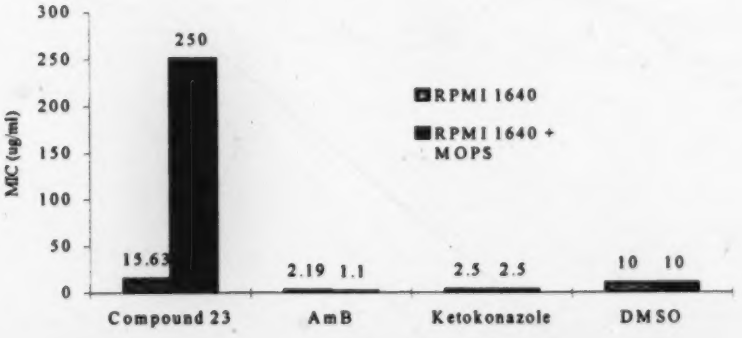


Figure 6-2. MIC values of compound 23 against *C. albicans* at 30°C in RPMI with and without MOPS buffer, compared to two standard antifungal agents; inoculum 3.3×10^3 (CFU/ml), incubation period 24 h; DMSO values are expressed as %v/v.

The other observation was that compound 23, angelicin and compound 61 show antagonism with the morpholine compound, MOPS. The antifungal activity of compound 23 against *C. albicans* has been greatly antagonized at 30° and 37° C by MOPS (Fig. 6-2 and 6-3). Interaction of AmB and ketoconazole with MOPS is in favor of antifungal agents, as they show stronger activity while MOPS is present. The effect of DMSO does not show any change in the presence or absence of MOPS. A similar result obtained with *C. neoformans* grown at 30°C, in which the presence of MOPS increased the MIC for compound 23 and decreased the MIC for ketoconazole.

This is the first report of such an interaction between MOPS and antifungal agents. The nature of this interaction is not quite clear, but it might be related to the biosynthetic pathway of ergosterol. As was described in the introductory chapter, morpholine compounds affect the ergosterol biosynthesis at two steps. Since no interaction between MOPS and DMSO has been detected, it is possible that the interference of activity in the presence of MOPS is a specific type limited to compounds affecting ergosterol biosynthesis.

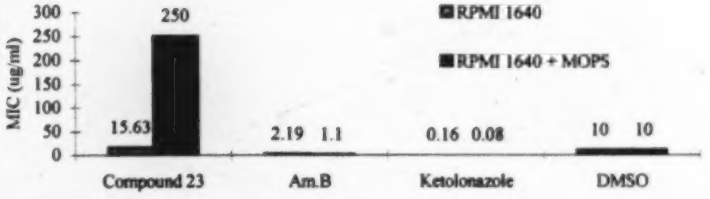


Figure 6-3. MIC values of compound 23 against *C. albicans* at 37°C in RPMI without and with MOPS buffer, compared to two standard antifungal agents, inoculum 3.3×10^3 (CFU/ml); incubation period 24 h. DMSO MIC values are expressed as %v/v.

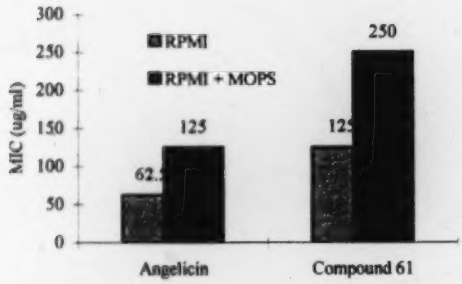


Figure 6-4. MIC values of angelicin and compound 61 in RPMI 1640 with and without MOPS against *C. albicans,* inoculum 3.3×10^3 (CFU/ml), grown at 37° C, incubation period 24 h.

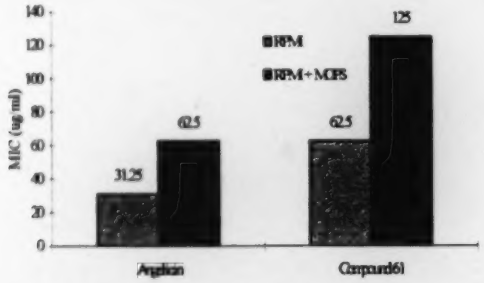


Figure 6-5. MIC values of angelicin and compound 61 in RPMI 1640 with and without MOPS against *Cryptococcus neoformans,* inoculum 3.3×10^3 (CFU/ml), grown at 37° C, incubation period 48 h.

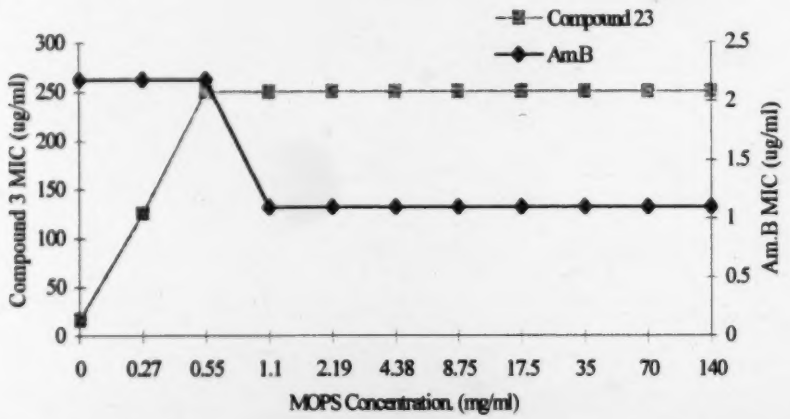


Figure 6-6. Relationship between MOPS concentration and MIC of compound 23 against *C. albicans* in RPMI 1640; incubation at 30° C for 24 h; inoculum 5×10^3 (CFU/ml).

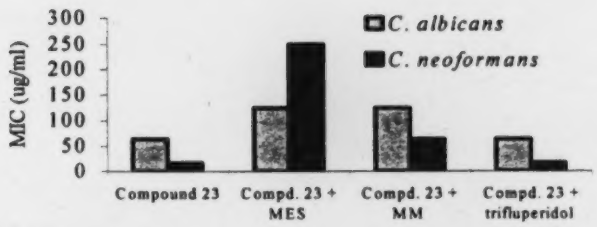


Figure 6-7. Changes in MIC value of compound 23 due to different morpholine compounds MES (250 μg/ml), MM (250 μg/ml), and trofluperidol (morpholine like) (31.3 μg/ml). Medium RPMI, inoculum, 5×10^3 (CFU/ml), 37° C, incubation for 24-48 h. None of the morpholine compounds have antifungal activity at the tested concentrations.

Angelicin and compound 61 have been affected by MOPS in the same manner as compound 23 (Fig. 6-4). *C. neoformans* is another organism that was inhibited to a lower extent by angelicin and compound 61 in presence of MOPS (Fig. 6-5). The inhibition of antifungal activity of coumarin 23 exhibited by MOPS is dose-dependent (Fig. 6-6). An opposite trend of interaction with MOPS is observed for AmB.

The above observations were tried with other MOPS analogues such as MES, MM, and trifluperidol. The result is shown in Figure 6-7. As can be seen, a similar type of interaction is observed for MES and MM, but not for trifluperidol. This result suggests very specific structural requirements for the inhibitory activity, which has not been reported previously. Separate experiments were performed to examine the possibility of physicochemical interaction of compound 23 with MOPS and other ingredients present in the medium (data not shown). However, chromatographic and UV spectrophotometric studies of the co-incubation product of these ingredients were not indicative of any transformation in the nature of the compounds. This is again supportive of the specificity of the antagonism of antifungal action of coumarins by morpholines tested at cellular level.

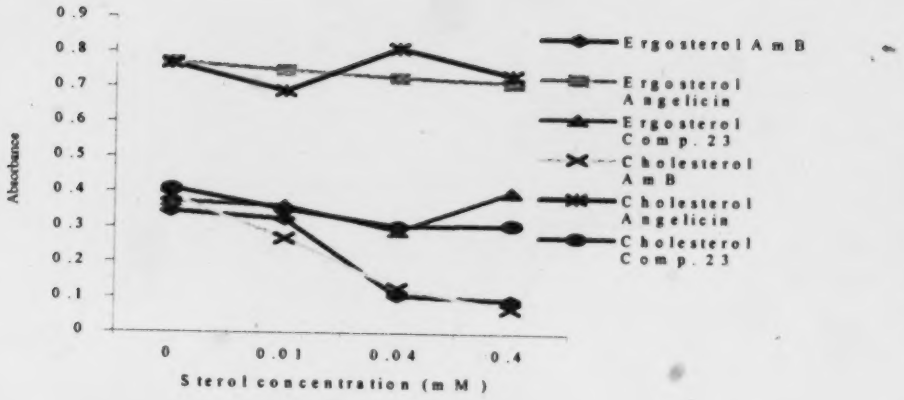


Figure 6-8. The interaction of ergosterol and cholesterol with three antifungal agents, as indicated by the UV absorption study. Am B, angelicin and compound 23 were applied at concentrations, 1.8, 12.5 and 6.25 μg/ml, and measured at wavelengths of 409, 302 and 317 nm respectively.

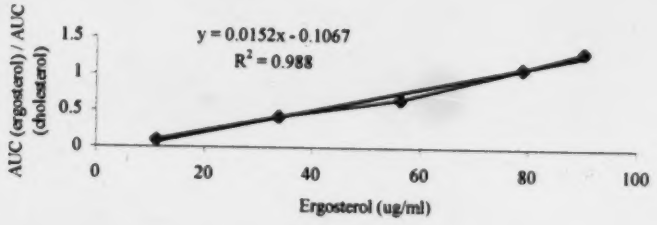


Figure 6-9. GC calibration curve for ergosterol using cholesterol as the internal standard.

To study the possible interaction of angelicin and compound 23 with ergosterol in a more detailed manner, a binding assay experiment was planned. In this test, the UV absorption of the compounds mixed with increasing concentrations of ergosterol and cholesterol in a hydro-alcoholic solution was measured at their proper λ_{max}. The absorption of AmB shows a significant decrease as the concentration of sterols increase (Fig. 6-8). This decrease in absorption has been attributed to the binding of AmB to sterols, which leaves less and less free form of AmB with absorption at 409 nm (Mazerki, *et al.*, 1995). In fact,

a red shift has been observed in the spectrum of AmB mixed with sterols, which is due to the binding with sterols. However, in the case of the coumarins, the decrease in the absorption is very small and is not concentration dependent. In addition, no change in the λ_{max} values of angelicin and compound 23 were observed after addition of sterols. An NMR study of mixed antifungal-sterol in d6-DMSO: d4-MeOH (1:1) solution was also carried out. The results did not show any dramatic change in the absorption frequency of the peaks, more than 1 Hz, for AmB-ergosterol and coumarin-ergosterol mixtures. Therefore, ^{1}H-NMR was not considered a powerful tool for detecting such intermolecular interactions.

Since many of the important antifungal drugs like azoles act on the ergosterol biosynthetic pathway and inhibit ergosterol biosynthesis at the 14-demethylation step, it was intended to evaluate the effect of coumarins on this metabolic pathway. As GC has a good resolving power and is accurate enough to measure ergosterol, using cholesterol as internal standard, (Fig. 6-9), it was selected for the quantitative evaluation of ergosterol. The assignment of peaks of sterols extracted from *C. albicans* and detected by GC (Fig. 6-10), was confirmed by comparing with the R_t value of authentic sterol samples and GC-MS analysis. The early peak, which is the biggest in the chromatogram of extracted sterols, is due to the solvent, acetone. The GC chromatogram of sterol fractions extracted from *C. albicans* treated with angelicin, compound 61, fluconazole, and db-cAMP are shown in Figures 6-11 to 6-14, respectively. Azoles like fluconazole are known to inhibit ergosterol biosynthesis and cause accumulation of sterols such as lanosterol, the product before 14-demethylation step. Therefore, new peaks appeared close to the ergosterol peak in the fluconazole GC chromatograph (Fig. 6-13). Yet, such a change was not observed in coumarin and c-AMP treated groups (Fig. 6-11, 6-12, and 6-14).

The cellular values measured for ergosterol in *C. albicans* treated with different coumarins are represented in Figure 6-15. As is shown in all the cases, the relative amount of ergosterol in treated groups is higher than the control. The increased ergosterol level is particularly visible in the angelicin group. The calculated values of ergosterol are based on the dried cellular mass. This value has to be less than control if the cells are

treated with a standard drug, which blocks the biosynthesis of ergosterol. According to Figure 6-16, the fluconazole treated group has much less ergosterol than control, which is an expected observation. The interesting increase in the group treated with the cyclic AMP analogue, db-cAMP, triggered a new set of experiments leading to a better picture of what is happening in the cell after applying coumarins.

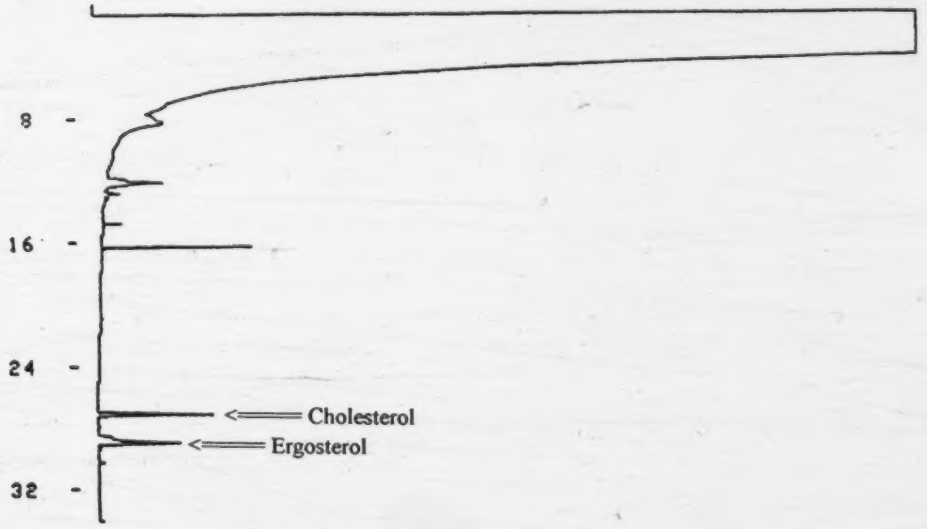


Figure 6-10. GC chromatogram of sterol fraction extracted from *C. albicans* grown at 30^0 C, using a 3% OV-1 column. The sterols are trimethylsilylated, and cholesterol is the internal standard.

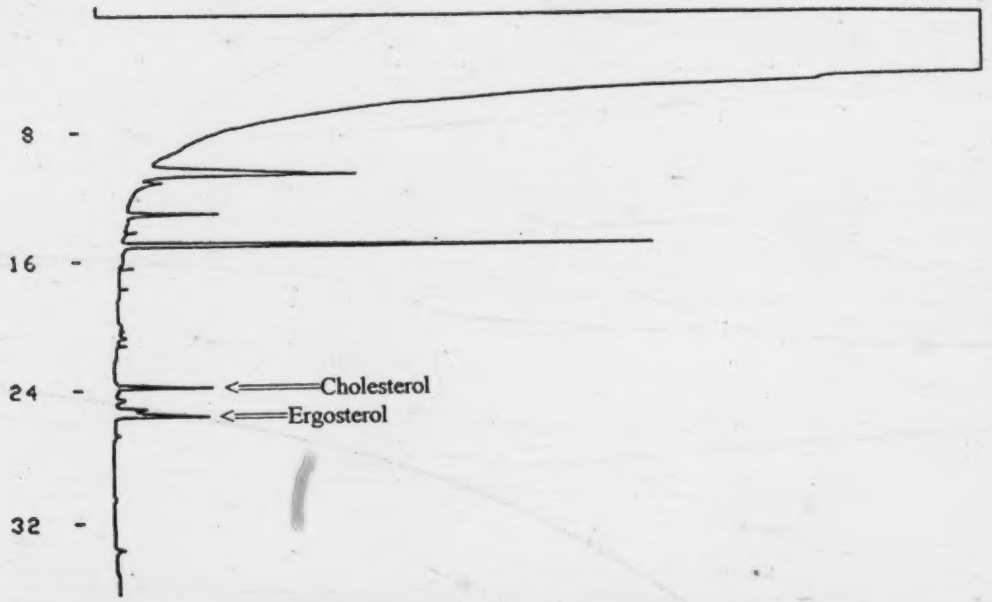


Figure 6-11. Typical GC chromatogram of sterol fraction extracted from *C. albicans* grown in the presence of angelicin (30 μg/ml) at 30^0 C, using a 3% OV-1 column. The sterols are trimethylsilylated, and cholesterol is the internal standard.

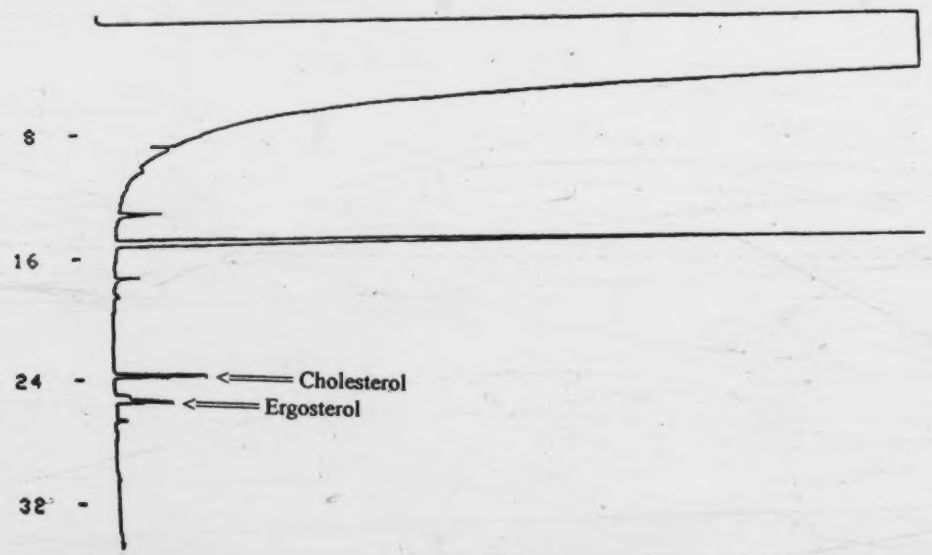


Figure 6-12. GC chromatogram of sterol fraction extracted from *C. albicans* grown in the presence of compound 61 (125 μg/ml) at 30^0 C, using a 3% OV-1 column. The sterols are trimethylsilylated, and cholesterol is the internal standard.

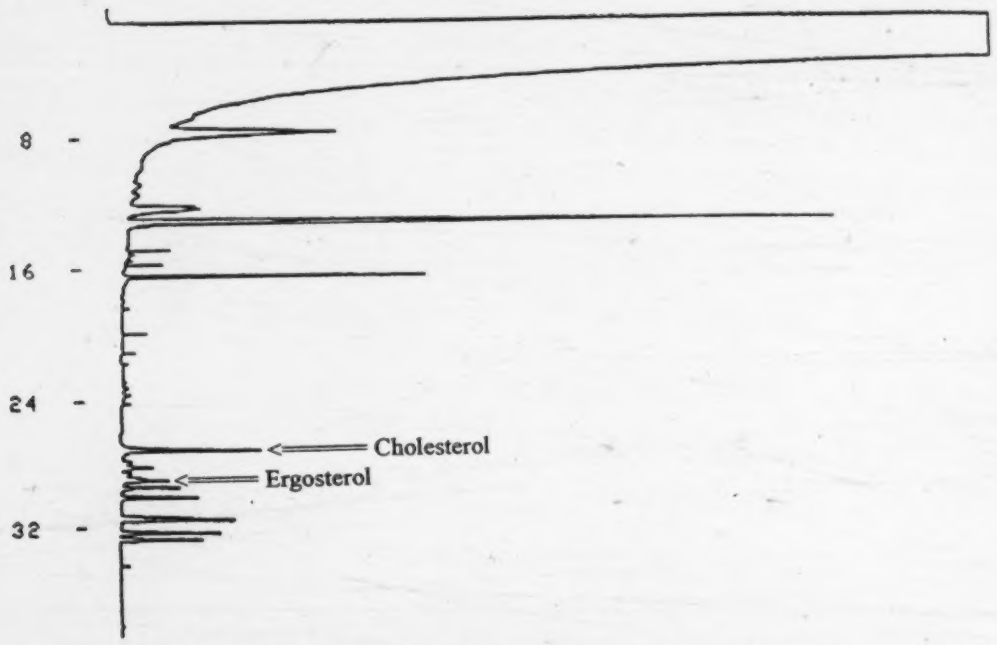


Figure 6-13. GC chromatogram of sterol fraction extracted from *C. albicans* grown in the presence of fluconazole (1 μg/ml) at 30^0 C, using a 3%OV-1 column. The sterols are trimethylsilylated, and cholesterol is the internal standard.

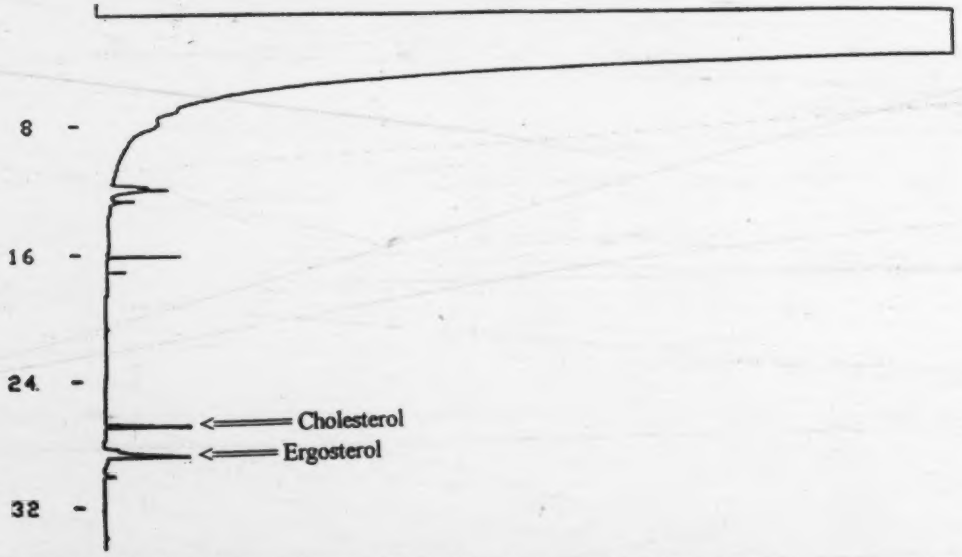


Figure 6-14. GC chromatogram of sterol fraction extracted from *C. albicans* grwon in the presence of db-cAMP (0.26 μM) at 30^0 C, using a 3% OV-1 column. The sterols are trimethylsilylated, and cholesterol is the internal standard.

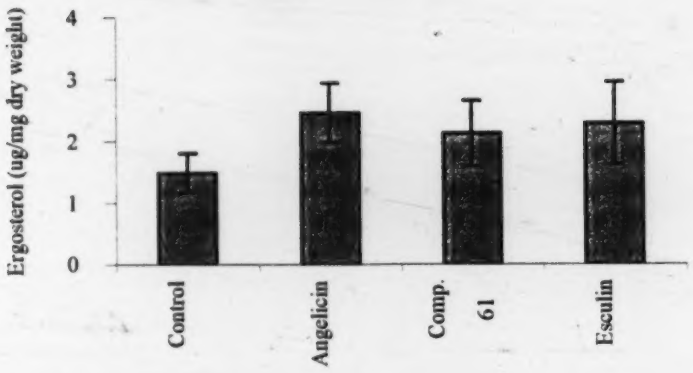


Figure 6-15. Effect of different compounds on the ergosterol content of *C. albicans* (n = 3, +/- SE). Cells were incubated in RPMI 1640, at 30°C for 24 h; concentrations of angelicin (30 μg/ml), compound 61 (125 μg/ml), esculin (500 μg/ml) were used.

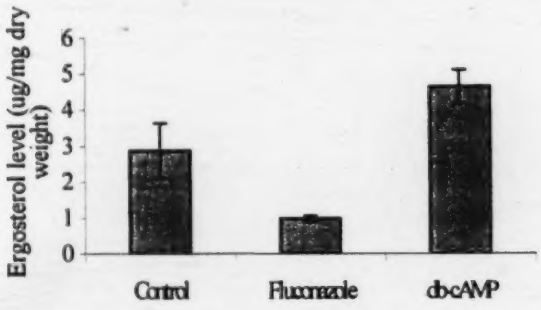


Figure 6-16. Ergosterol level in *C. albicans* treated with fluconazole and db-cAMP (n = 3, +/-SE). Cells were incubated in RPMI 1640, at 30°C for 24 h; concentrations of fluconazole (1 μg/ml), db-cAMP (128 μg/ml) were used.

The effect of db-cAMP on the MIC values of three antifungal drugs, ketoconazole, fluconazole and AmB against *C. albicans* and *C. neoformans* is shown in Figures 6-17 and 18. All of the MIC values for both fungi have been shifted to higher degrees. This result was reproducible in the applied concentration of db-cAMP, and the rest of the microwells showed more growth than the wells containing only antifungal agent. The adenosine derivative db-cAMP was not inhibitory by itself. The growth curve of *C. albicans* in the presence of fluconazole, db-cAMP and their mixture is presented in

Figure 6-19. The result again is consistent with interaction studies and suggesting that the antifungal activity of azoles and AmB are being reduced by cAMP derivative added to the cell culture media.

In order to find out the effect of the intracellular level of cAMP on the antifungal activity of drugs, the agents that are inhibitory to phosphodiesterase, the enzyme responsible for degradation of cAMP in the cell were studied. For this purpose, several phosphodiesterase inhibitors (PDIs) were chosen, including theophylline, pentoxyfylline, and IBMX. As it is shown in Figure 6-20, the inhibitory action of ketoconazole on *C. albicans* and *C. neoformans* has been reduced by all PDIs. The largest antagonism effect was observed in the theophylline group. The susceptibility of *C. neoformans* and *C. albicans* has generally been reduced to azole antifungals (Figures 6-21 and 6-22). In the case of AmB no change in MIC value was observed for *C. neoformans*, however by increasing the incubation period for *C. albicans* the differences become quite obvious as is shown in Fig 6-22.

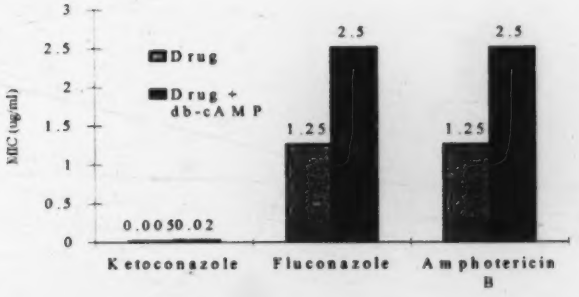


Figure 6-17. MIC values of several antifungal drugs alone and in combination with db-cAMP (max. 250 µg/ml), against *C. albicans*. Medium was RPMI 1640, incubated at 37° C, for 48h incubation.

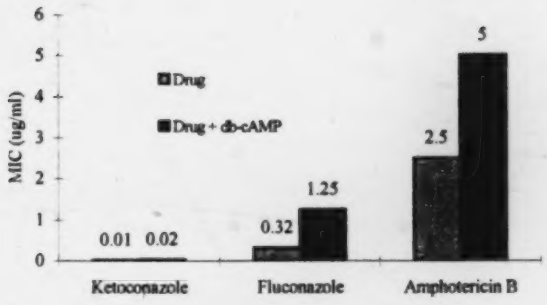


Figure 6-18. MIC values of several antifungal drugs alone and in combination with db-cAMP (max. 250 μg/ml), against *C. neoformans*. Medium was RPMI 1640, incubated at 37° C, for 48h.

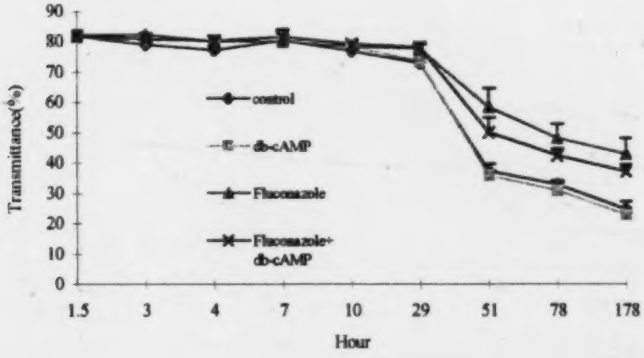


Figure 6-19. Growth curve of *C. albicans* in the presence of fluconazole and db-cAMP; in RPMI 1640, 37°C; concentrations of fluconazole (0.31μg/ml), db-cAMP (125 μg/ml) and inoculum, 5×10^3 (CFU/ml) were used (n = 3, +/- SE). Fungi were pre-incubated with db-cAMP, 1 h before addition of fluconazole.

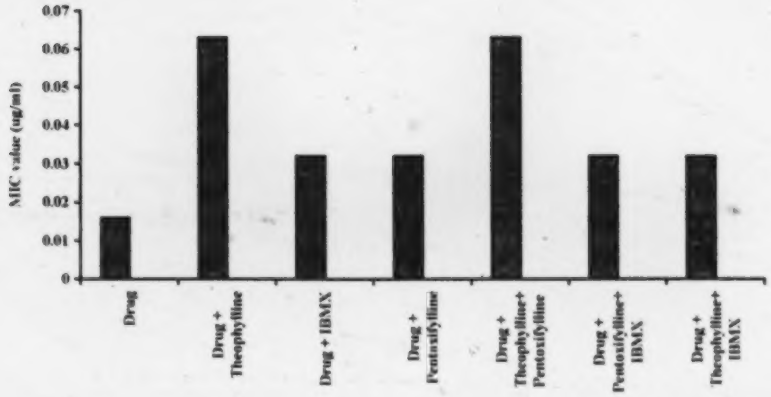


Figure 6-20. Changes in the MIC value of ketoconazole due to co-incubation with different phosphodiesterase inhibitors (PDIs). Organism *C. neoformans* (Pentoxifylline group tested on *C. albicans* with a ketoconazole MIC of 0.008 µg/ml). Medium RPMI, with MOPS incubated at 37°C for 48 h, concentrations of PDIs were as follows: theophylline (1000 µg/ml), IBMX (100 µg/ml), pentoxifylline (25 µg/ml). PDIs were not inhibitory at the concentrations used.

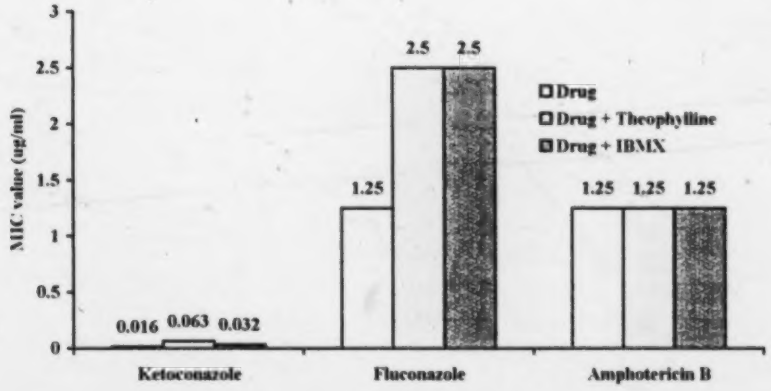


Figure 6-21. Changes in the MIC value of several antifungals due to co-incubation with different phosphodiesterase inhibitors. Organism, *C. neoformans*; medium RPMI, with MOPS incubated at 37°C, for 48 h, concentrations of PDIs were as follows: theophylline (1000 µg/ml), IBMX (100 µg/ml), pentoxifylline (25 µg/ml). PDIs were not inhibitory at the concentrations used.

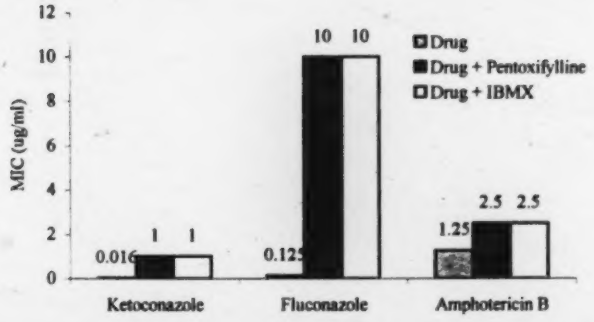


Figure 6-22. Changes in the MIC value of several antifungals due to co-incubation with different phosphodiesterase inhibitors. Organism, *C. albicans*; medium RPMI, with MOPS incubated at 37°C for 96 h, concentrations of PDIs were as follows: IBMX (10 μg/ml), pentoxifylline (25 μg/ml). PDIs were not inhibitory at the concentrations used.

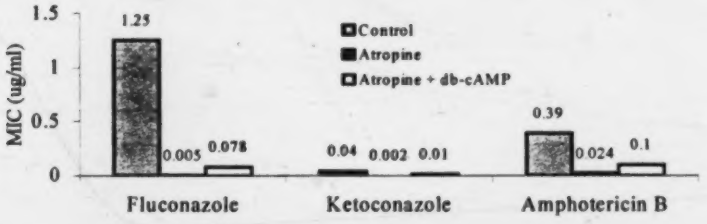


Figure 6-23. Changes in the MIC value of several antifungals due to co-incubation with db-cAMP (250 μg/ml) and adenylate cyclase inhibitor, atropine (125 μg/ml). Organism *C. neoformans*, medium RPMI incubated for 48 h at 37°C. Atropine was not inhibitory to the fungi at the concentration tested.

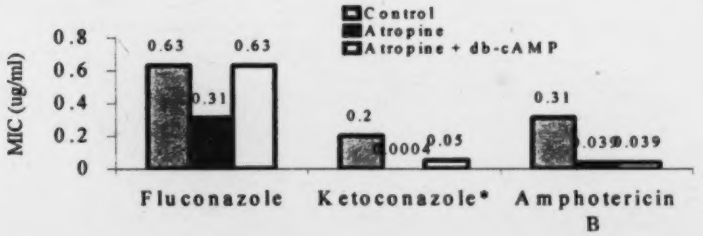


Figure 6-24. Changes in the MIC value of several antifungals due to co-incubation with db-cAMP (250 μg/ml) and adenylate cyclase inhibitor, atropine (250 μg/ml). Organism *C. albicans*, medium RPMI incubated for 24 h at 37°C. Atropine was not inhibitory to the fungi at the concentration tested. *Ketoconazole values are 100×MIC.

In addition to the above-mentioned observations regarding the involvement of intracellular cAMP levels in ergosterol metabolism, more supporting facts were found in our further experiments. Another enzyme that regulates cellular cAMP level is adenylate cyclase, AC, which unlike phosphodiesterase is involved in the synthesis of cAMP from ATP. It was shown earlier that external cAMP analogues could increase the ergosterol level and inhibit the antifungal action of drugs acting on ergosterol metabolism, and also PDIs can act like the cAMP analogue in this regard. By reducing the cAMP level, it should be possible to observe a synergistic effect of antifungals. This possibility was investigated using atropine, which is a blocker of adenylate cyclase, ACI. In Figures 6-23 and 6-24, the synergism between atropine and antifungal drugs is clearly seen for both *C. neoformans* and *C. albicans*. The activity of atropine, as anticipated, has been partially or completely reversed by using a cAMP analogue.

Since the action of coumarin compounds in antagonizing azole antifungals, and increasing the ergosterol level of fungal cell were similar to db-cAMP, and in spite of no structural similarity between cAMP and coumarins, it was hypothesized that coumarins should increase the cAMP level of the fungal cell. In order to evaluate this theory, the phosphodiesterase inhibition test assay was performed on a number of natural and synthetic coumarins. The results are shown in Table 6-1. In comparison to caffeine, which is a standard PDI, it can be seen that most of the coumarins tested show strong inhibitory activity on phosphodiesterase. Among the simple coumarins, 4, and 21-22, the iodo derivative is the strongest enzyme inhibitor. Among the heavier molecular weight derivatives, 47-51, and 53, compound 50 is clearly superior. Trisubstituted coumarins 56 and 54, both show strong PDI activity, however, 54 is the stronger derivative. Although many of the furocoumarins, 28, 61, 27, 26, 31, 33, 35, and 59 have moderate to strong inhibitory activity, 61 and 59 are the strongest and 35 is not active. From the table, it becomes clear that antifungal and phosphodiesterase activity do not necessarily follow each other. In fact, the picture is more complicated than it seems.

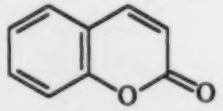
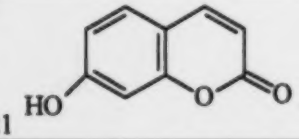
Two factors could be important for a coumarin to show phosphodiesterase inhibition. First is the lipophilicity. A comparison among the following similar pairs revealed that

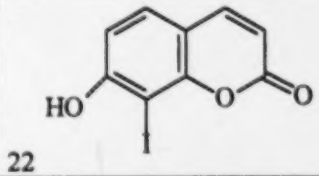
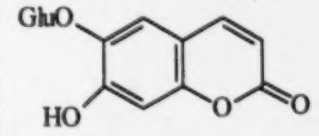
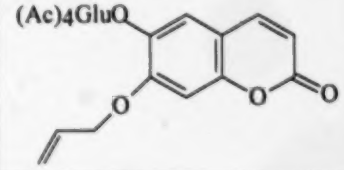
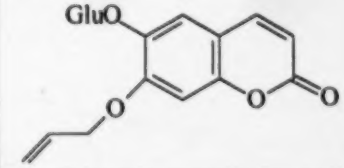
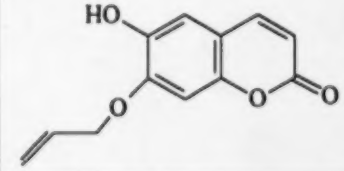
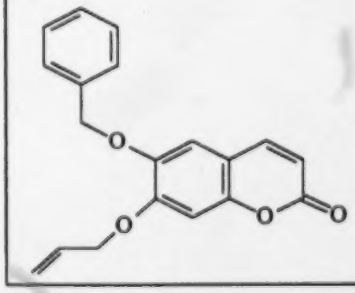
the more lipophilic one is usually the stronger inhibitor (mentioned in brackets): 21 and 22 (22), 49 and 48 (49), 48 and 50 (50), 51 and 53 (51), 56 and 54 (54), 27 and 26 (26), 31 and 33 (33).

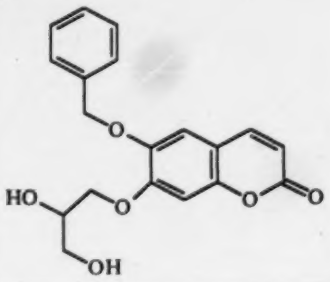
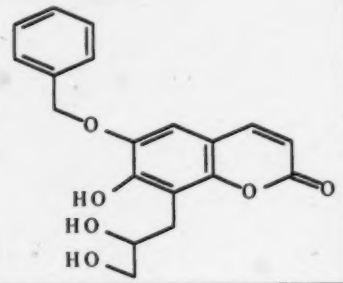
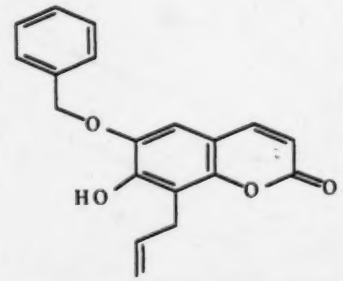
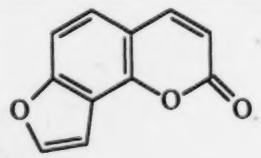
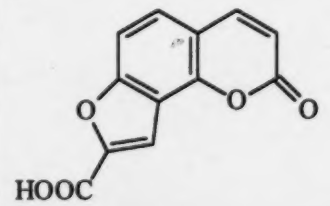
The second factor is the presence of a phenolic hydroxyl group. Representations of this group are the following pairs of coumarins, in which the stronger inhibitor of each pair has one free phenolic hydroxyl (in brackets): 4 and 21 (21), 48 and 50 (50), 28 and 59 (59). It should be noted that the strongest inhibitors of all coumarins tested, i.e., compounds 54 and 59, have both factors that is high lipophilicity and one phenolic hydroxyl group. The position of hydroxyl group can be variable at either location 6 or 7. There are certain compounds with phenolic hydroxyl but showing very weak or no activity. The example in this series is compounds 47, which is a weak inhibitor because the glucose substituent makes it too polar to show any activity.

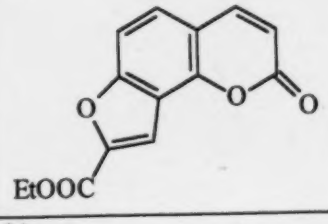
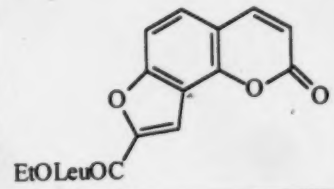
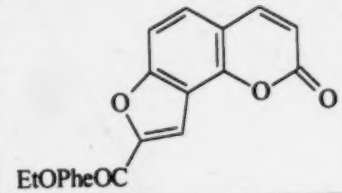
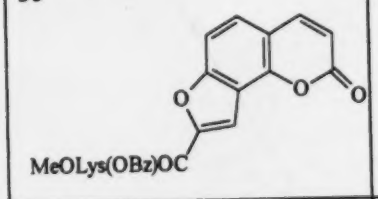
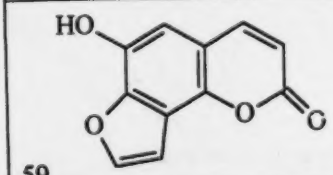
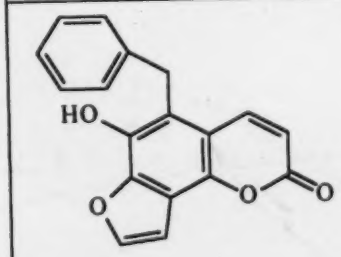
The other point that should considered is the enzyme, which was obtained from bovine heart. This brings up the possibility of variation with fungal phosphodiesterase. Therefore, not all of the relative and absolute IC_{50} values for the PDI activity of coumarins mentioned in Table 6-1 have to be the same for the fungal enzyme.

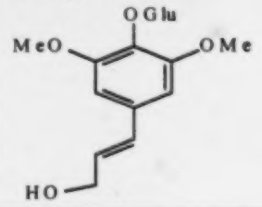
Table 6-1. cAMP Phosphodiesterase (bovine heart muscle) inhibitory activity of several coumarin derivatives.

Compound	PDI activity ($IC_{50} \times 10^{1}\mu M$)	Antifungal activity (MIC $\times 10^{-1}\mu M$)		
		C. albicans	*C. neoformans*	*S. cerevisiae*
 4	178.58	>684.93	342.47	684.93
 21	40.34	617.3	308.6	Nt

 22	5.77	347.2	173.6	Nt
 47 Esculin	>500	>294.98	>294.98	>294.98
49 	36.66	>182.48	>182.48	>182.48
48 	>500	>263.16	>263.16	>263.16
50 	11.23	>458.72	114.68	114.68
51 	54.92	>324.68	>324.68	>324.68

53 	121.56	>147.06	73.53	73.53
56 	13.50	>292.4	292.4	292.4
54 	2.90	>324.68	162.34	324.68
 28 Angelicin	31.60	33.6	134.41	67.2
27 	25.33	>869.6	869.6	Nt

26 	22.71	387.6	96.9	Nt
31 	22.38	>269.5	269.5	Nt
33 	15.34	>493.8	30.9	246.9
35 	-	>197.6	>197.6	>197.6
 59	4.71	123.76	61.88	123.76
 61	7.47	171.23	42.81	171.23

Syringin 	-	>268.8	>268.8	>268.8
Caffeine	58.6			

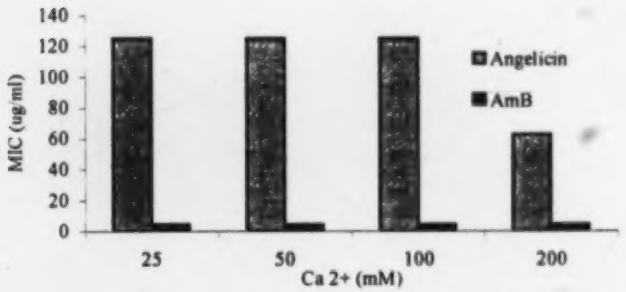


Figure 6-25. Ion susceptibility of *C. albicans* to calcium, added as $CaCl_2$, in the presence of angelicin, compared to AmB; medium RPMI 1640, incubation at 30° C for 24h, inoculum 3.3×10^3 (CFU/ml).

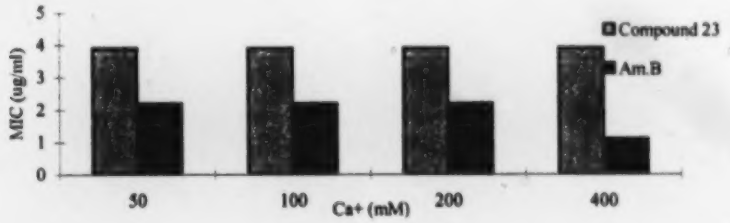


Figure 6-26. Effect of different calcium concentrations, added as $CaCl_2$, on the MIC of compound 23 against *C. albicans*; medium RPMI, incubation at 30° C for 24h, inoculum 3.3×10^3 (CFU/ml).

The state of plasma cell membrane may change due to effect of antifungal compounds. Azoles, by inhibiting ergosterol synthesis and AmB through specific binding to

membrane ergosterol and subsequent deformations in the membrane, cause leakage of nutrients like amino acids and minerals from the cell. Increase in cell membrane permeability can cause penetration of ions into the cell along their gradient force.

A simple test for determination of plasma membrane integrity is to expose the cell to higher than normal concentrations of ions and observe their subsequent toxicity to the cell. The result of exposure of *C. albicans* to Ca^{2+} in the presence of antifungal agents is shown in Figures 6-25 and 6-26. Angelicin has been able to increase the sensitivity of fungal cells at 200 mM of Ca^{2+}, while AmB at 400 mM and compound 23 perhaps needs higher concentrations.

A similar test was carried out using Na^{+} gradient. In this case, angelicin imposes an increased sensitivity at lowest Na^{+} level, while AmB and compound 23 both indicate potentiation of inhibition as the concentration of Na^{+} is increased (Figures 6-27 to 6-29). In all of the above-mentioned tests, cells grew normally in the presence of high ion concentrations; therefore the osmolarity of the medium is not playing a role by itself.

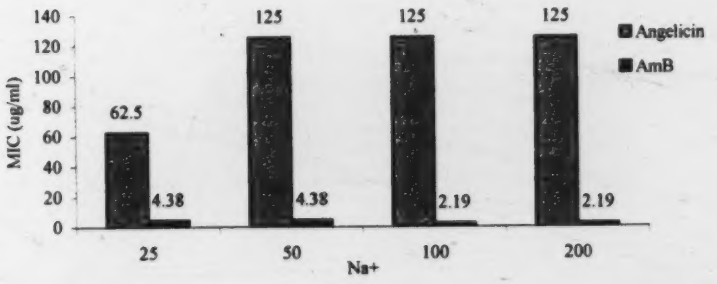


Figure 6-27. Ion susceptibility test of *C. albicans* in the presence angelicin, compared to AmB in RPMI incubated at 30° C for 24 h, inoculum 3.3×10^{3} (CFU/ml).

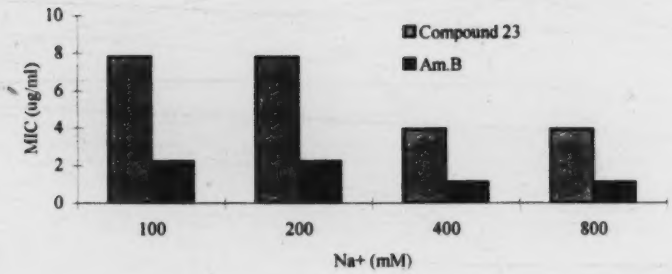


Figure 6-28. Effect of different sodium concentrations, added as NaCl, on MIC of compound 23 towards *C. albicans*; medium RPMI, incubation at 30° C for 24h, inoculum 3.3×10^3 (CFU/ml).

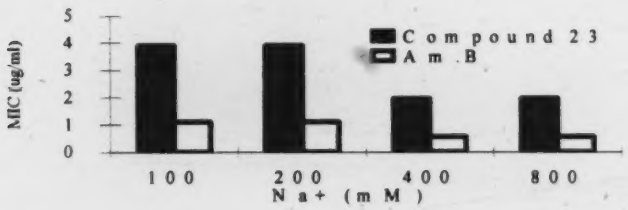


Figure 6-29. Effect of sodium ion concentration, added as NaCl, on the MIC of compound 23 against *S. cerevisiae* grown in RPMI, at 30° C for 48 h, inoculum 3.3×10^3 (CFU/ml).

Effect on H^+-ATPase: It was out of curiosity that we decided to determine the reason for buffer sensitivity of MIC in the coumarin group of antifungals. One of the simple tests that was carried out in this regard was changing the buffer and pH and observing the variations in MIC values. The results are shown in Figure 6-30. Compound 23 has a lower MIC in medium containing phosphate buffer at pH 7, compared to the medium buffered with MOPS (Fig. 6-2). At the same time, reducing the pH to 6 or removing the buffer altogether increased the activity dramatically. The drug or control groups show no change or decrease in activity at the lower pH. Since AmB is an unstable molecule, light and acid sensitive, and the fact that many fungi grow better in the slightly acidic pH, may

explain the increase in the MIC value of AmB. Ketoconazole and DMSO MICs are unaffected by the changes in pH (Figures 6-30 and 6-31).

The interesting results obtained encouraged us to test more hypotheses in this regard. The inhibitory activity of compound 23 and angelicin is shown to be pH-dependent; antifungal activity increases at lower pH values (Figures 6-31 to 6-33). This phenomenon was not observed in ketoconazole and DMSO groups, which could be interpreted as a specific effect not related to damage to the membrane.

Since the fungal cell adjust its interior pH by outwards pumping of protons through the action of membrane bound H^+-ATPase, damage to the cell membrane could mean inwards penetration of H^+ and consequent cytotoxicity due to intracellular pH change. Azole drugs cause a general disturbance by decreasing ergosterol and increasing concentration of lanosterol in the fungal cell membrane. However, ketoconazole did not show any pH-dependency in its activity. Therefore, gradient-forced penetration of protons into the fungal cell should not be happening.

At this point the mechanism of pH-dependent activity of coumarin compounds becomes of interest. In order to investigate the above phenomenon further, reviewing the possible scenarios could be quite helpful. Since the intracellular pH of fungal cell is about 7.4, there is an inward gradient of protons which helps co-transport of nutrients like amino acids into the cell. Therefore, any decrease in the intracellular pH or increase in the extracellular pH could be harmful for the fungal cell, since many kinds of nutrients cannot be imported into the cell. The pH-dependency of omeprazole (a H^+-ATPase inhibitor) action (Fig. 6-33) and synergism of compound 23 with other agents affecting ATPase enzymes (Figures 6-34 and 6-35), like ouabain and Na_3VO_4, or cell membrane potential, like hygromycin B and valinomycin, is indicative of the fact that coumarins may change the cell membrane potential possibly through inhibition of H^+-ATPase.

Since the main system for controlling cellular pH and membrane potential in fungal cells is the action of H^+-ATPase, its blockage can lead to serious interruptions in cell functions

and cause abnormalities such as sensitivity to weak acids. The acetate loading sensitivity of *C. albicans* in the presence of angelicin and compound 23 (Fig. 6-36), supports this idea.

One of the side effects of inhibiting H^+-ATPase in the fungal cell would be lower acid secreting capacity, which translates into higher medium pH. Measurement of medium acidification can be easily carried out by fast responding pH meters. However, a better method is to use NMR to examine the peak positions of phosphorous in the extra- and intracellular environment. Since the peak position of phosphorous is pH-dependent (Fig. 6-37), it can be used to track down the changes in pH values *in vivo* and even estimate the pH value.

By looking at Figures 6-38, it becomes clear that the chemical shift of Pi(int) peak in *C. albicans* cells treated with angelicin is moved upfield. This trend in the displacement of peak position is corresponding to a more acidic cytoplasm. The results of glucose induced medium acidification is shown in Figure 6-39. In this case, the cytoplasmic acidity of the angelicin treated group is again more than the control group, as represented by an upfield shift of Pi(int) peak. In addition, acidification of the medium is much less than the control group, as indicated by a less upfield shift in the Pi(ext) peak position. These experiments all support the hypothesis that H^+-ATPase is inhibited and the cell cannot pump out the protons generated by metabolism, thus causing intracellular pH to drop. Polyphosphate peak in Figure 6-39 (PP4-n) shows a big shift too. Since most of the polyphosphates is located in the vacuole, this is indicative of a change in the vacuole pH. In fact, there is special H^+-ATPase located in the vacuole membrane, which help in adjusting intracellular pH. This enzyme pumps protons into the vacuole and the resulting co-transport will transfer some nutrients into the vacuole. Since the pH of the vacuole has been changed, it is possible that vacuolar H^+-ATPase has also been inhibited by coumarins. Figure 6-40 shows that compound 23 has an even larger inhibitory effect on medium acidification by *C. albicans*.

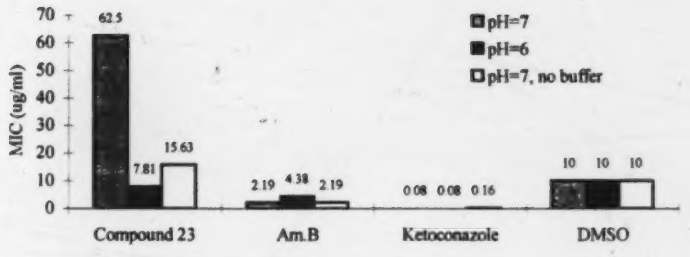


Figure 6-30. Sensitivity of *C. albicans* to compound 23 in RPMI 1640, using phosphate buffer; incubation at 30°C, for 24 h, inoculum 5×10^3 (CFU/ml). DMSO MIC value has been expressed as %v/v.

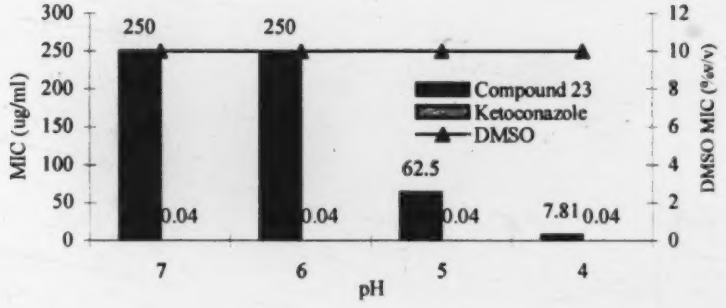


Figure 6-31. Variation of MIC value of compound 23 with pH against *C. albicans* grown at 30° C in RPMI 1640 containing MOPS, for 24 h, inoculum 3.3×10^3 (CFU/ml).

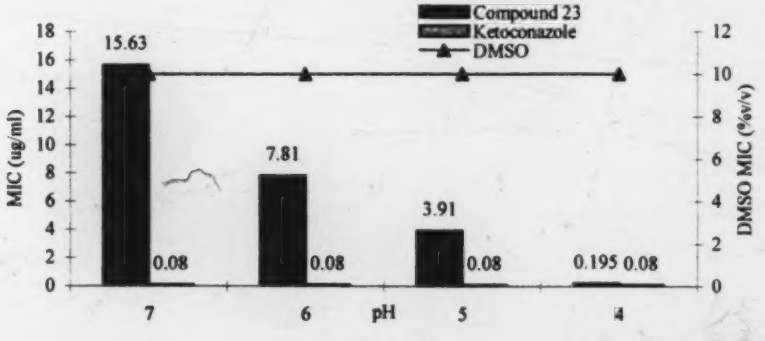


Figure 6-32. Variation of MIC value of compound 23 with pH against *C. albicans* grown at 30° C in RPMI 1640 without addition of MOPS for 24 h, inoculum 3.3×10^3 (CFU/ml).

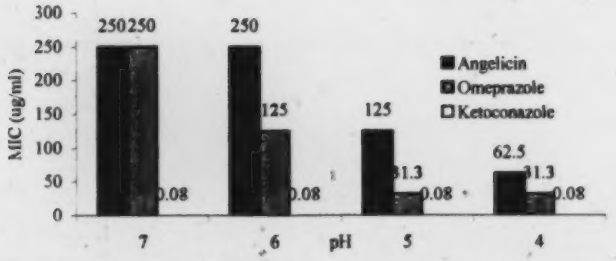


Figure 6-33. Variation of MIC value of angelicin with pH against *C. albicans* in RPMI 1640 without addition of MOPS grown at 37° C for 24 h, inoculum 5×10^3 (CFU/ml). Ketoconazole values are 10×MIC.

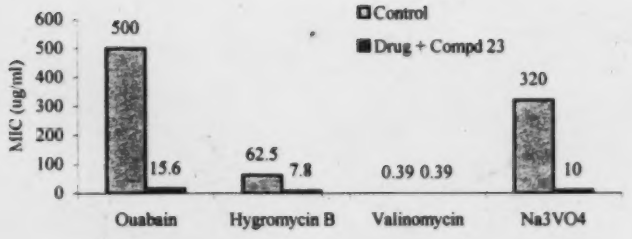


Figure 6-34. Variation of MIC values (μg/ml) of selected ATPase modulating compounds in broth dilution test against *C. albicans* with and without co-incubation with compound 23. Medium, RPMI 1640, pH=7, concentration of compound 23, 3.9 (μg/ml); temperature 37°C; incubation period 24 h; inoculum, 5×10^3 (CFU/ml).

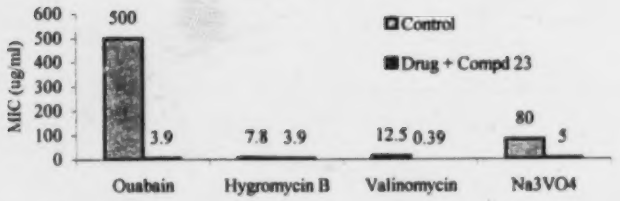


Figure 6-35. Variation of MIC values (μg/ml) of selected ATPase modulating compounds in broth dilution test against *C. neoformans* with and without co-incubation with compound 23. Medium, RPMI 1640, pH=7, concentration of compound 23, 3.9 (μg/ml); temperature 37°C; incubation period 48 h; inoculum, 5×10^3 (CFU/ml).

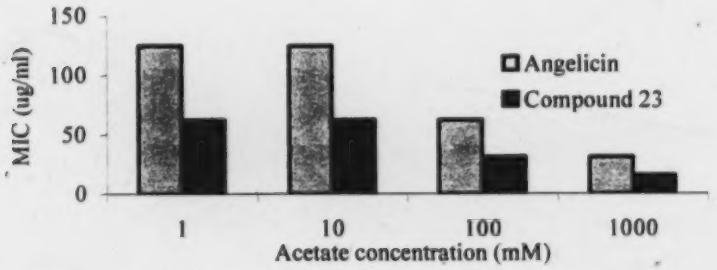


Figure 6-36. The acid loading sensitivity of *C. albicans* to angelicin and compound 23 in the presence of different concentrations of sodium acetate. Medium RPMI, pH = 7, incubated at 37°C for 24 h, inoculum 5×10^3 CFU/ml.

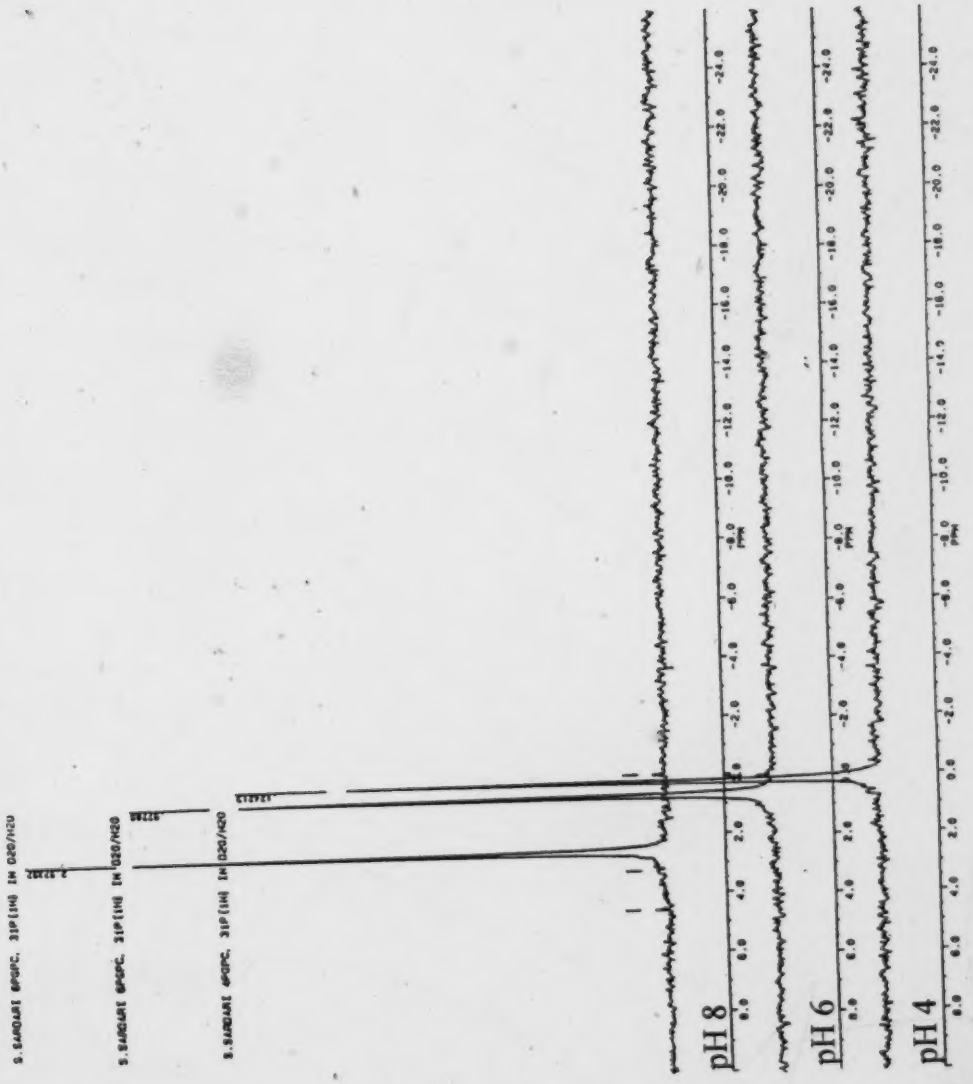


Figure 6-37. ^{31}P-NMR of Glycerol phosphorylcholine in RPMI (+P) at different pH values.

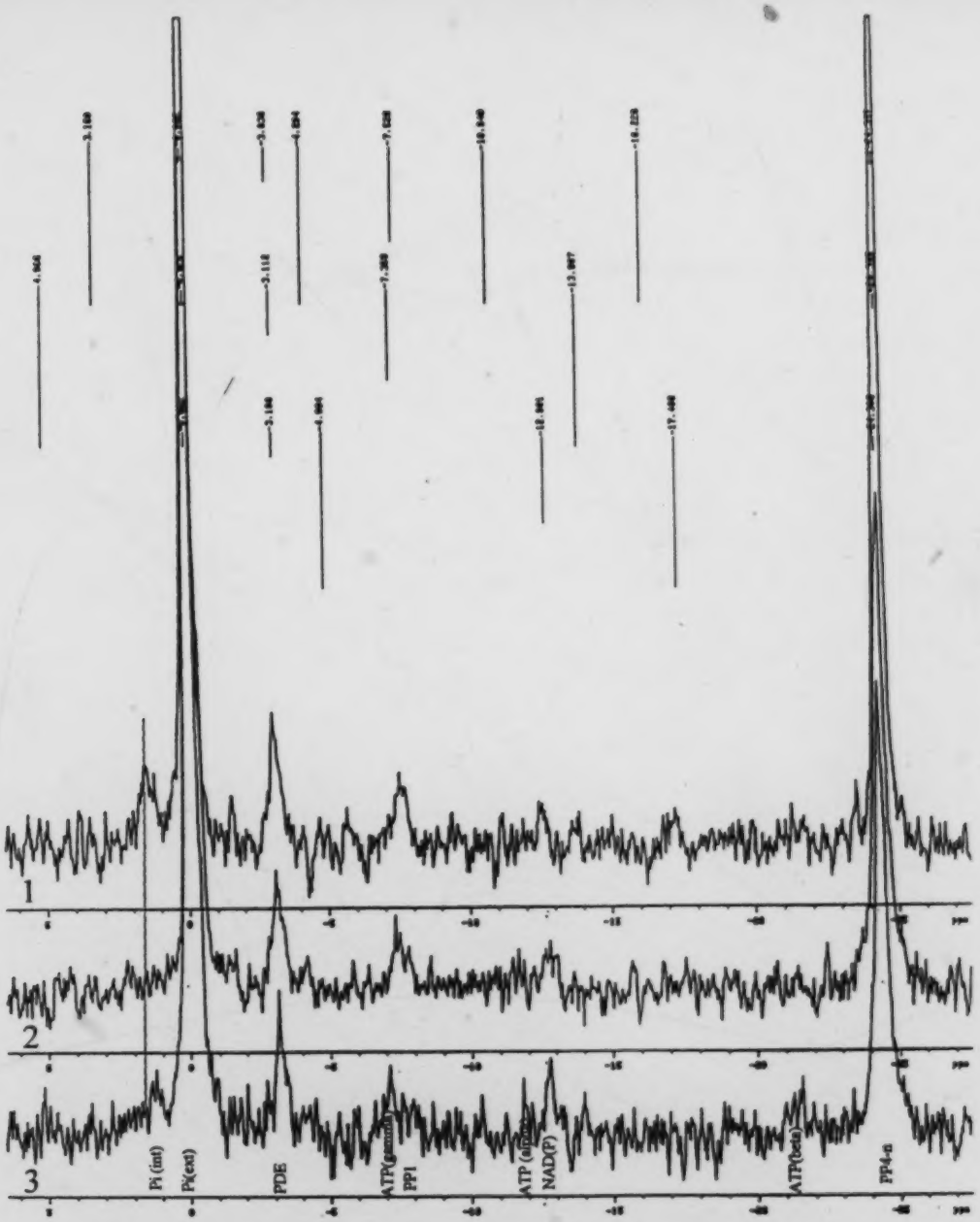


Figure 6-38. ^{31}P-NMR spectra of *C. albicans* cells grown in RPMI (+P) at 25^0 C, (1) control group, (2) solvent control (methanol), (3) angelicin administered group. Pi(int), inorganic phosphate intracellular; Pi(ext), inorganic phosphate extracellular; PDE, phosphodiester peak from cell wall components; ATP (γ), the gamma phosphorous of ATP; PP1, terminal phosphorous of polyphosphates; ATP (α), alpha phosphorous of ATP; NAD(P), phosphorous peak of NADP; ATP(β), beta phosphorous of ATP; PP4-n, long chain vacuolar polyphosphate. The vertical line cutting the spectra is aligned with the chemical shift of control group spectrum.

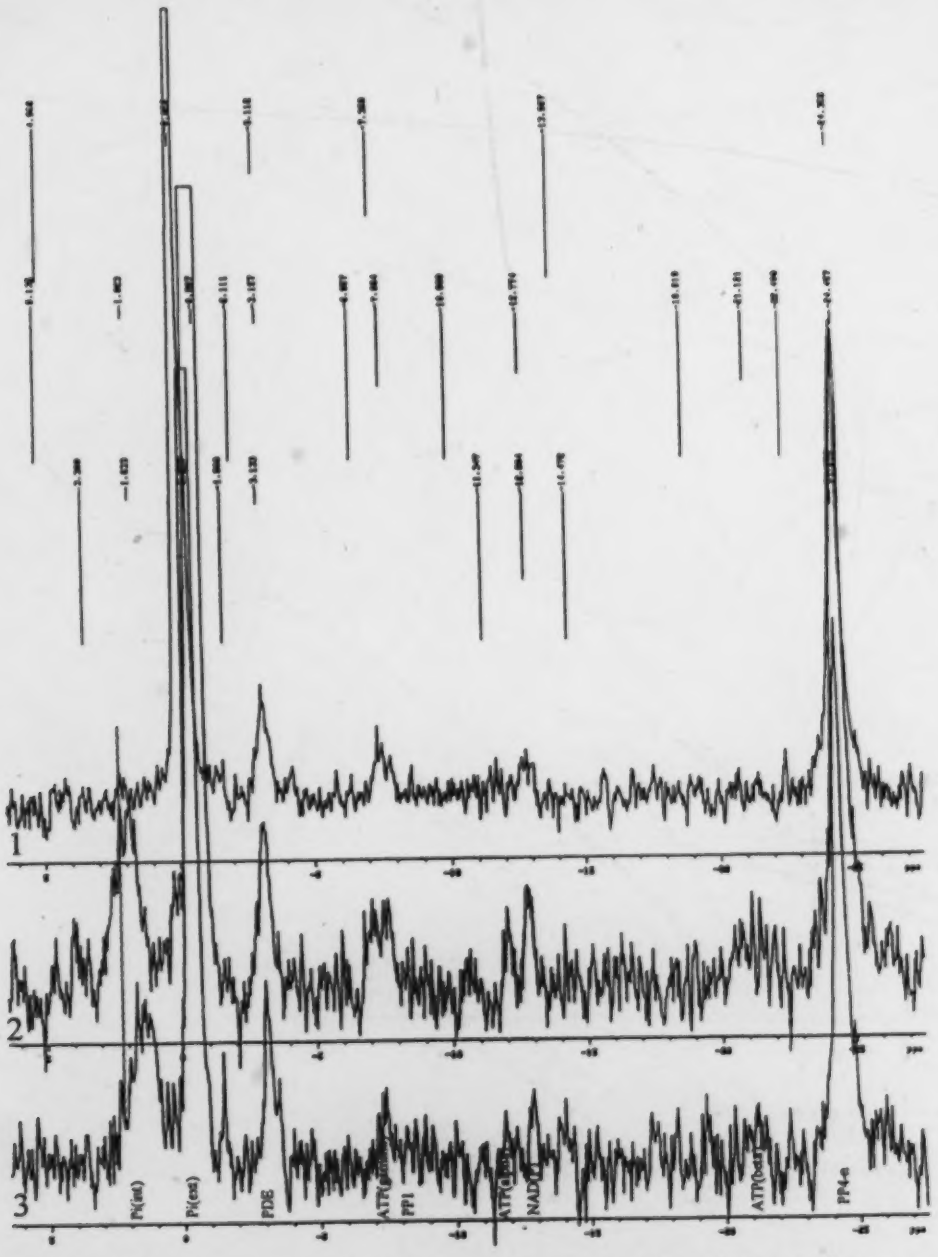


Figure 6-39. ^{31}P-NMR spectra of *C. albicans* cells grown in RPMI (+P) at 25^0 C, (1) solvent (methanol) control group, (2) solvent group with glucose, (3) angelicin treated with glucose. Pi(int), inorganic phosphate intracellular; Pi(ext), inorganic phosphate extracellular; PDE, phosphodiester peak from cell wall components; ATP (γ), the gamma phosphorous of ATP; PP1, terminal phosphorous of polyphosphates; ATP (α), alpha phosphorous of ATP; NAD(P), phosphorous peak of NADP; ATP(β), beta phosphorous of ATP; PP4-n, long chain vacuolar polyphosphate. The vertical line cutting the spectra is aligned with the chemical shift of control group spectrum.

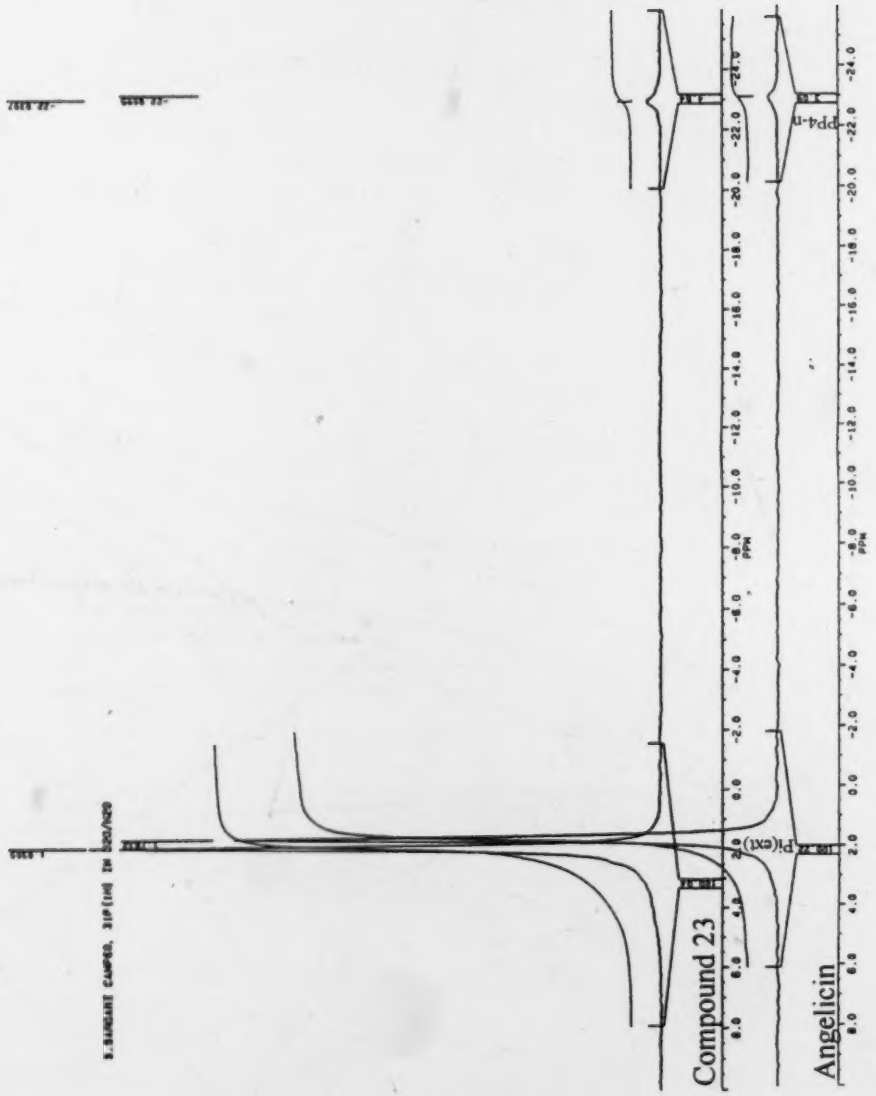


Figure 6-40. ^{31}P-NMR of *C. albicans* treated with angelicin and compound 23. Growth condition: medium RPMI (+P), temperature 25^0 C, incubation period 20 h.

Morphological changes:

Electron microscopic examination of fungal cells is a powerful mean of studying morphological changes. Scanning electron microscopy (SEM) is a technique that is utilized to look at the surface of organism. SEM pictures of *C. albicans* cells treated with angelicin, compound 61 and 23, nitroangelicin, and AmB are shown in Figures 6-41 to 6-45. Although there is not a clear change of structure in the angelicin group, others show significant changes. As can be seen in Fig. 6-42 and 6-43, cells are in a phase of multibudding. In the cells treated with subinhibitory concentrations of compound 61, cell wall is rugged. A similar pattern is repeated in the cells co-incubated with low concentrations of nitroangelicin (Figure 6-44). However, the compound 23 and AmB groups have very few cells with a wrinkled surface. AmB group cells do not exhibit marked changes (Figure 6-45), as the MIC of AmB is very sharp and subinhibitory concentration practically does not trail to lower concentrations.

In transmittance electron microscopy (TEM) of the *Candida* cells (Figures 6-46 to 6-55), more details of intracellular changes are observed. The control cells are shown in Figures 6-46 to 6-49, show healthy cell wall, cell membrane, and in some cases, internal organelles such as nucleus and vacuole. However, by a quick look at the cells treated with angelicin (Figures 6-50 and 6-51) and compound 23 (Figures 6-52 and 6-53) two big changes are evident.

The first change can be observed in the vacuoles. It looks like the vacuolar membrane invaginates to form an intravacuolar membrane matrix that eventually breaks down, leaving clumps of densely stained and amorphous material surrounded by electron lucent areas. Invagination of vacuole may be cutting the vacuole into several smaller compartments. The second change is observed in the cell membrane, which is quite fuzzy in the angelicin group and thin or unclear in compound 23 group. The cells treated with a standard antifungal drug, fluconazole are shown in Figures 6-54 and 6-55. In this group, as expected, the cell membrane is disrupted and various abnormalities appear in the organelles.

Figure 6-41. SEM picture of *C. albicans* cells exposed to angelicin (7.8 μg/ml), grown in RPMI containing citrate buffer at 37° C for 24 h.

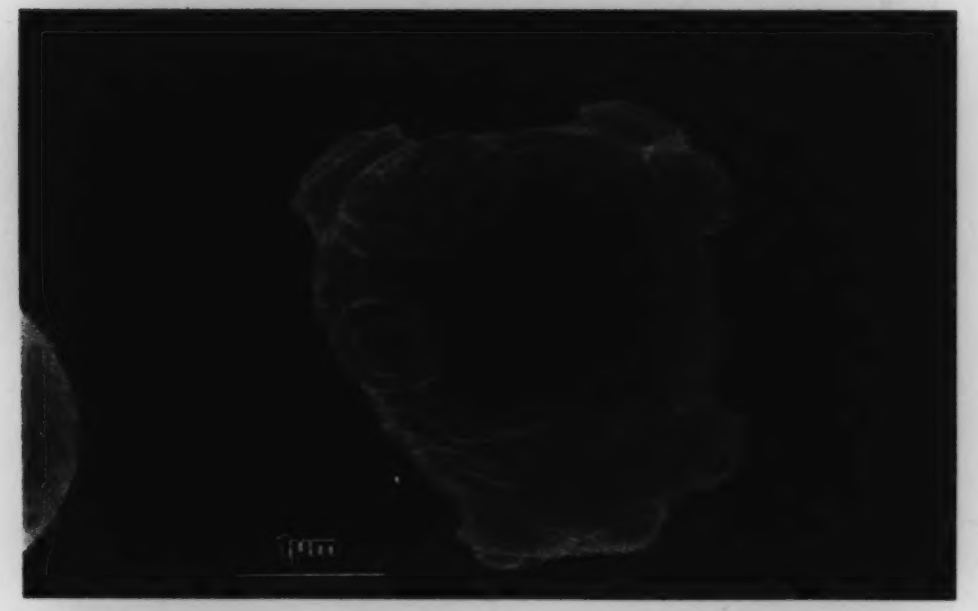


Figure 6-42. SEM picture of *C. albicans* cells exposed to compound 61 (15.6 μg/ml), grown in RPMI containing citrate buffer at 37° C for 24 h.

Figure 6-43. SEM picture of *C. albicans* cells exposed to compound 23 (3.9 μg/ml), grown in RPMI containing citrate buffer at 37° C for 24 h.

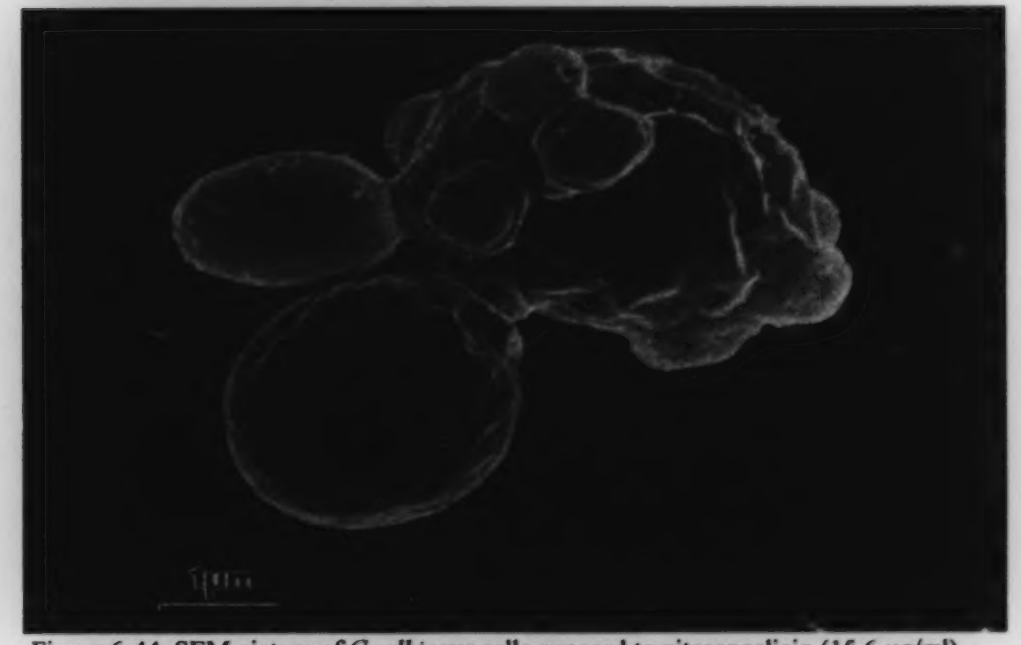


Figure 6-44. SEM picture of *C. albicans* cells exposed to nitroangelicin (15.6 μg/ml), grown in RPMI containing citrate buffer at 37° C for 24 h.

Figure 6-45. SEM picture of *C. albicans* cells exposed to AmB (2.5 μg/ml), grown in RPMI containing MOPS buffer at 37° C for 24 h.

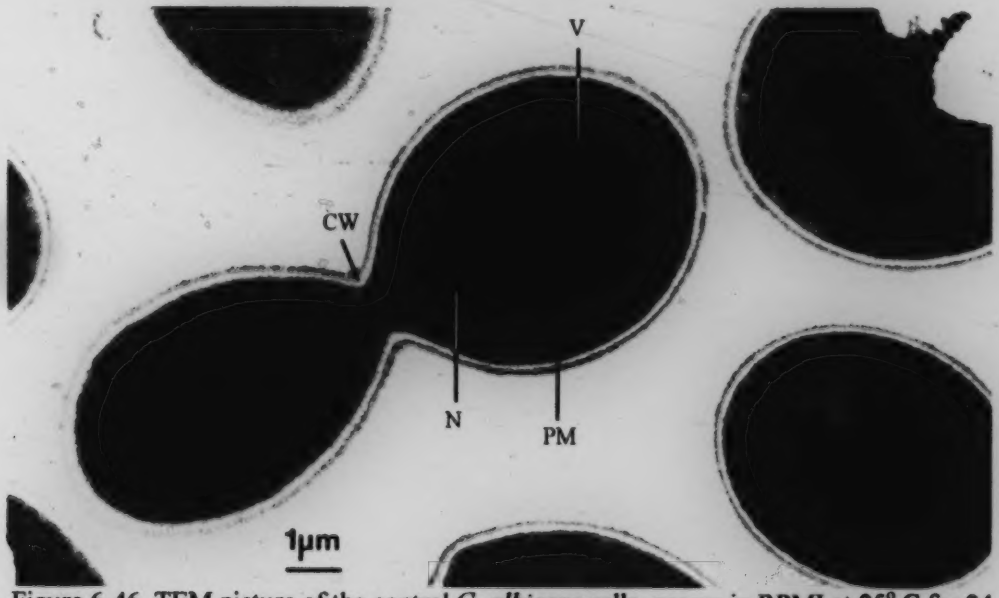


Figure 6-46. TEM picture of the control *C. albicans* cells, grown in RPMI at 25° C for 24 h.×2100. CW, cell wall; PM, plasma membrane; N, nucleus; V, vacuole.

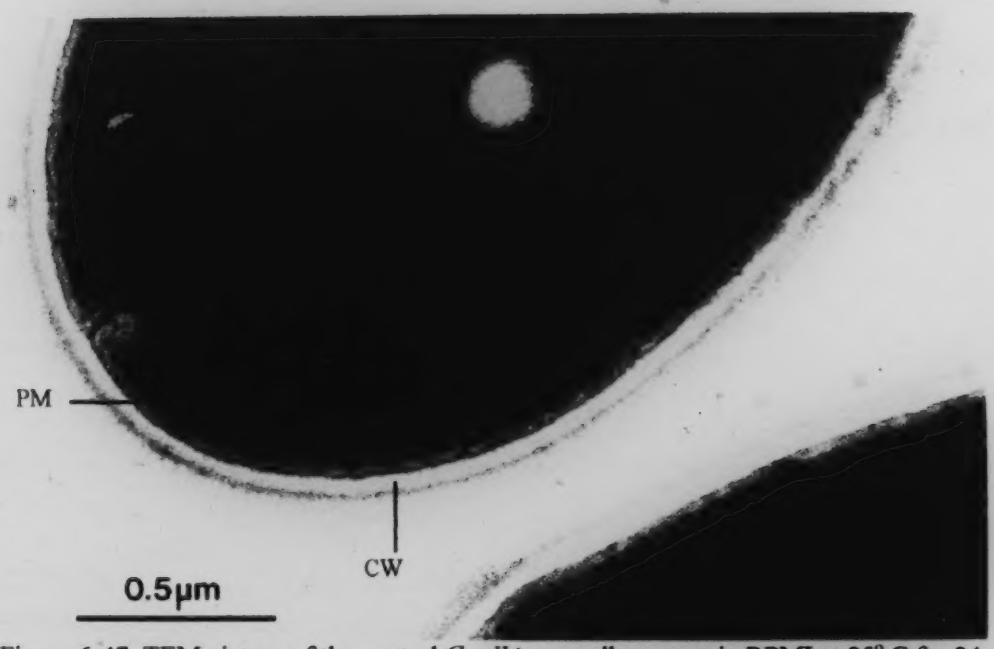


Figure 6-47. TEM picture of the control *C. albicans* cells, grown in RPMI at 25° C for 24 h.×12000. CW, cell wall; PM, plasma membrane.

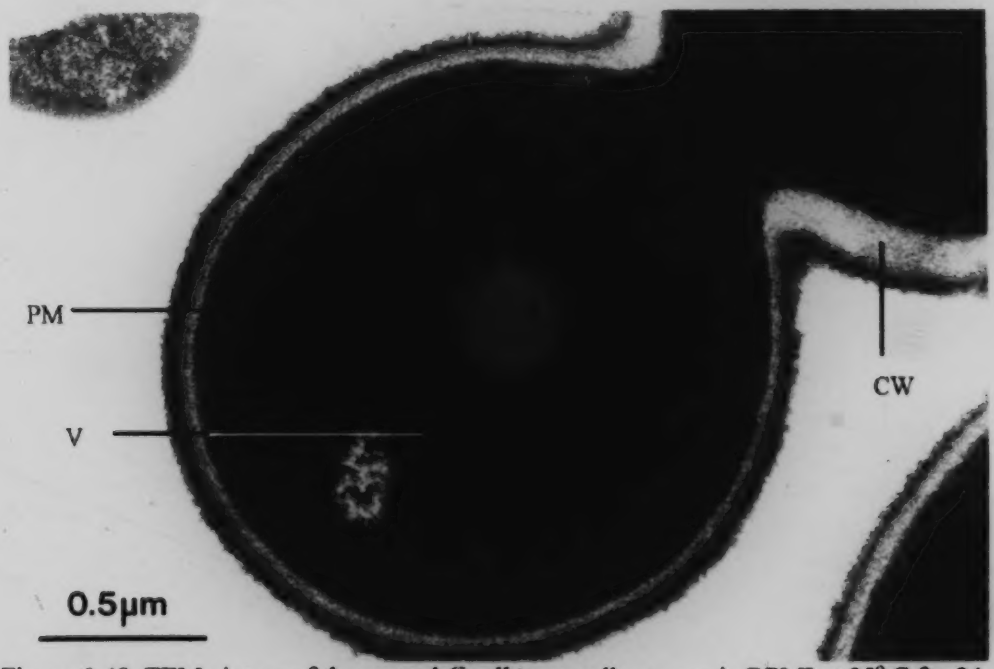


Figure 6-48. TEM picture of the control *C. albicans* cells, grown in RPMI at 25° C for 24 h.×3000. CW, cell wall; PM, plasma membrane; V, vacuole.

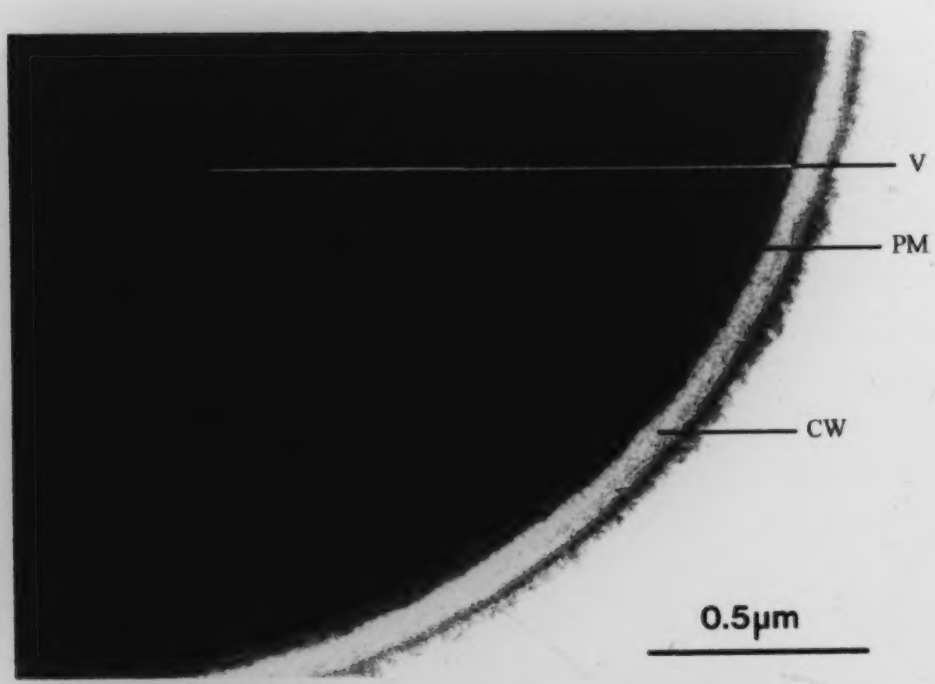


Figure 6-49. TEM picture of the control *C. albicans* cells, grown in RPMI at 25° C for 24 h.×40000. CW, cell wall; PM, plasma membrane; V, vacuole.

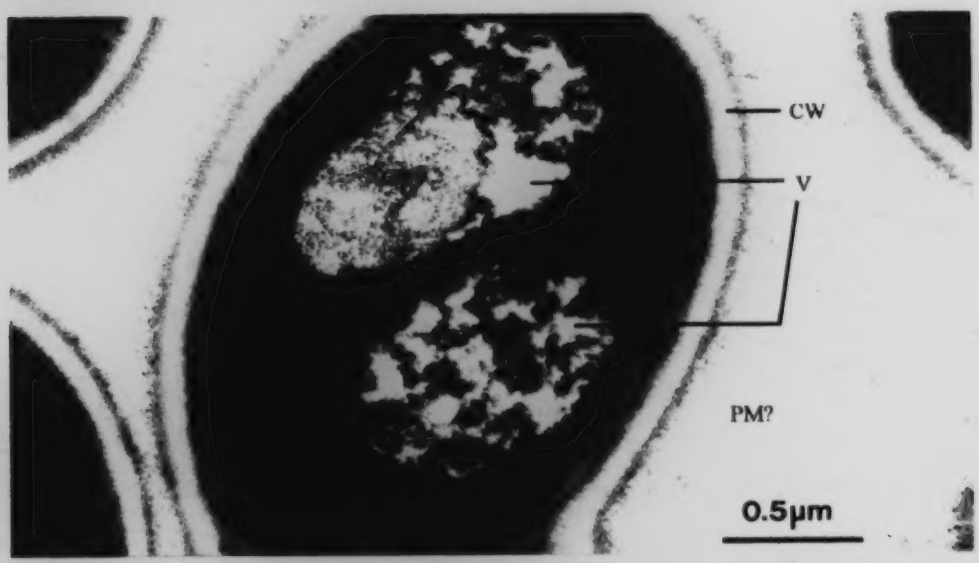


Figure 6-50. TEM picture of *C. albicans* cells, exposed to angelicin, grown in RPMI at 25° C for 24 h.×6000. CW, cell wall; PM, plasma membrane; V, vacuole.

Figure 6-51. TEM picture of *C. albicans* cells, exposed to angelicin, grown in RPMI at 25° C for 24 h.×9000. CW, cell wall; PM, plasma membrane; V, vacuole.

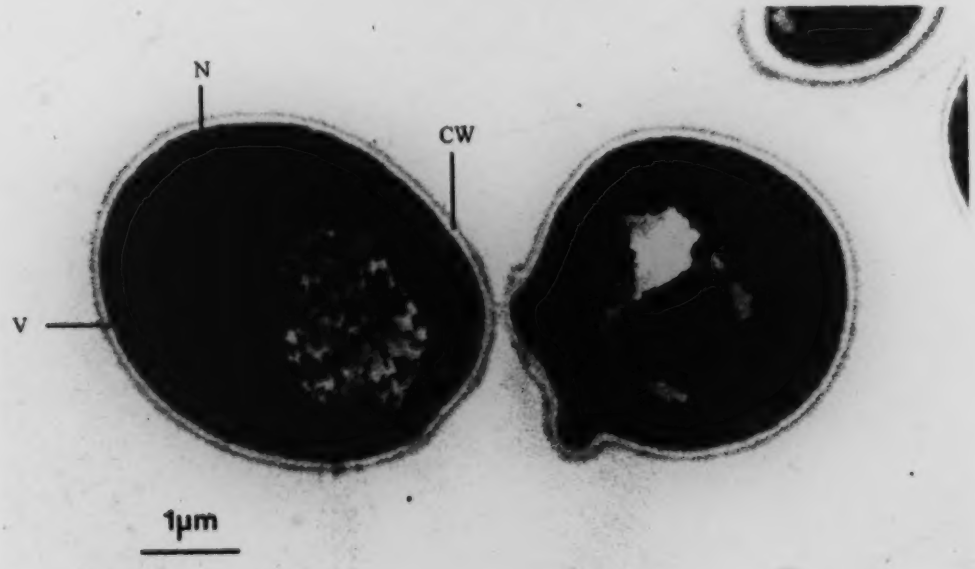


Figure 6-52. TEM picture of *C. albicans* cells, exposed to compound 23, grown in RPMI at 25° C for 24 h.×3000. CW, cell wall; PM, plasma membrane; V, vacuole.

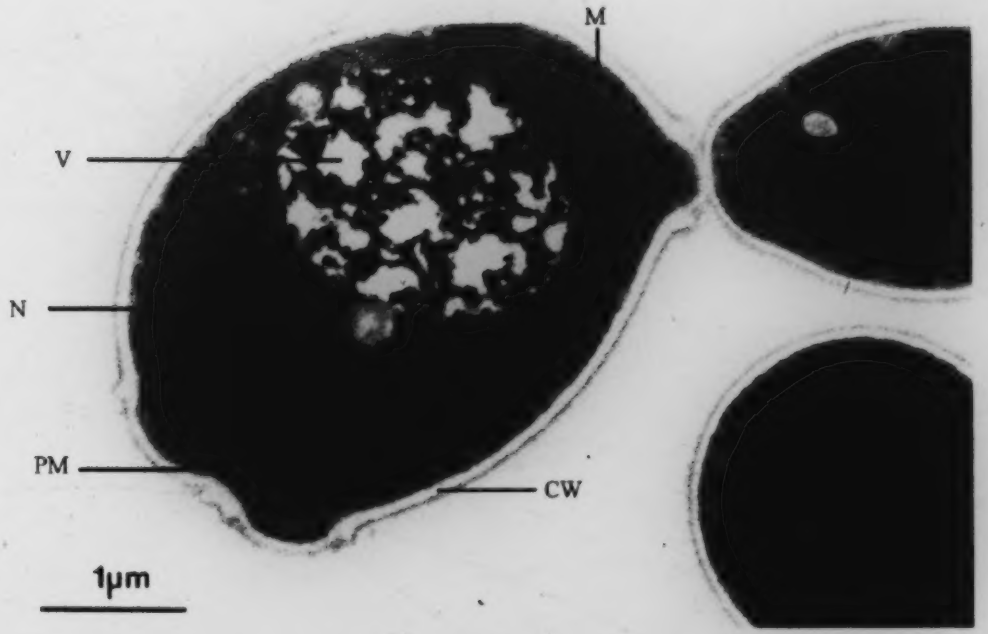


Figure 6-53. TEM picture of *C. albicans* cells, exposed to compound 23, grown in RPMI at 25° C for 24 h.×4500. CW, cell wall; PM, plasma membrane; N, nucleus; V, vacuole, M, mitochondria.

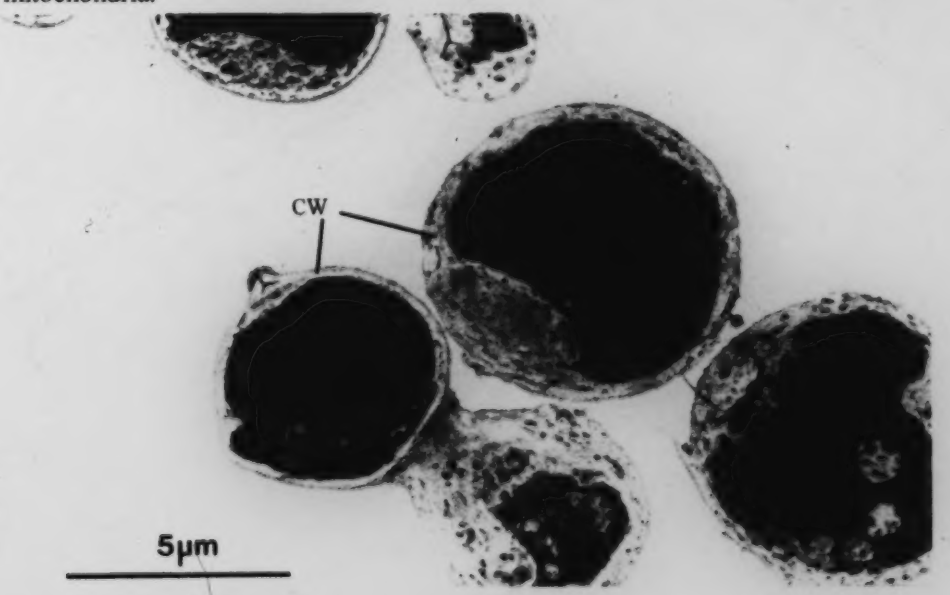


Figure 6-54. TEM picture of *C. albicans* cells, exposed to fluconazole, grown in RPMI (K_3PO_4, 2 mM) at 25° C for 24 h.×1500. CW, cell wall.

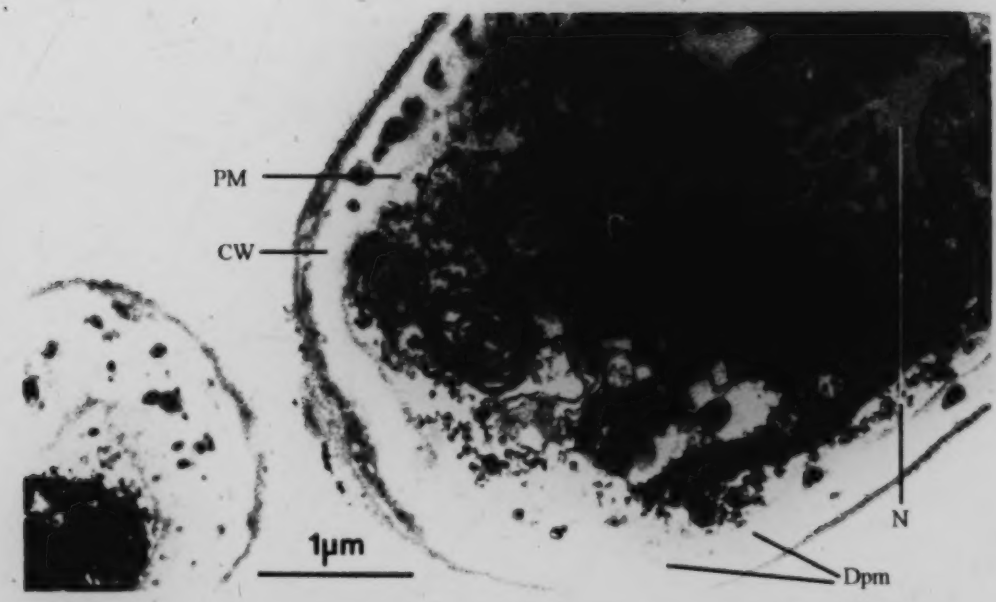


Figure 6-55. TEM picture of *C. albicans* cells, exposed to fluconazole, grown in RPMI (K_3PO_4, 2 mM) at 25° C for 24 h.×5100. CW, cell wall; PM, plasma membrane; N, nucleus; Dmp, damaged plasma membrane.

Discussion

Identifying the mechanisms of action of known drugs or new agents is an ongoing process in drug development. In many cases including known drugs, novel actions that are not identified before may be found and this could lead to refining of their therapeutic profile. One example is the azole antifungals, which show calmodulin modulating activity in addition to the known ergosterol biosynthesis inhibition (Hegemann, *et al.*, 1993)

Coumarins are shown to have many different bioactivities. Coumarin and its major human metabolite, 7-hydroxycoumarin, either as a single agent or in combination with immune modulators, have demonstrated significant antitumor activity without any noticeable symptomatic side effects (Keightley, *et al.*, 1996). Among the bioactivities of coumarins, photoreactivation has been the subject of many studies (Martin, *et al.*, 1966). However, only a few reports have studied other cellular processes which could be

involved in the cell inhibition caused by coumarins. It has been demonstrated that coumarin affect neither the cytoskeleton arrangement nor induce depolymerization of microtubules (Itoh, 1976).

Effect on ergosterol and cAMP:

Interaction studies are useful means of investigating the possible mode of action of antifungals. It is reported that the cells pre-exposed to azoles are protected from subsequent killing by short-time exposure to AmB. If cells are continuously exposed to AmB, the azoles must also be present continuously to maintain its protective effect. Also the cell physiology and stage of growth can affect susceptibility of fungi to drugs. For example, stationary-phase *Candida albicans* cells are more resistant to polyenes because of an increased level of β-1,3-D-glucans in the cell wall (Vazquez, *et al.*, 1996).

The antagonistic interaction observed between coumarin 23 and ketoconazole indicated that the mechanism of action should be quite different, but related to azoles. Synergism could be expected in the cases that two different points of a target have been attacked. An additive effect is assumed if two different agents have targeted the same location. The argument that the osmotic pressure might have contributed to the effect of MOPS on the activity of 23 would be ruled out by considering the results of testing different concentrations of Ca^{2+} and Na^{+} that did not affect or reduce the MIC of compound 23.

Ergosterol binding studies has been done on other natural antifungals. Saponins were shown to precipitate with ergosterol (Leconte, *et al.*, 1997). As a good precipitation method could not be established, UV spectroscopy was applied with reasonable results. The possibility of AmB-like activity as is observed for saponins was ruled out, due to weak interaction of coumarins with ergosterol and cholesterol.

Since oomycete fungi like *Pythium* do not have ergosterol in their membrane, they do not respond well to AmB (Sathapatayavongs, *et al.*, 1989). Our investigation on *Saprolegnia ferax* ATCC 46240 showed no activity for angelicin, compound 23 and AmB in disk

diffusion method. Therefore a complex non-specific mode of action, such as effect on protein synthesis, is not predicted for coumarins studied here. However, the involvement of ergosterol could still be considered.

Sterols, especially ergosterol, are very important for most fungal cells. Mutants defective in sterol transmethylation had a sixfold reduction in tryptophan uptake. Cholesterol, lanosterol, and ergosta-7,22-dienol could satisfy the bulk of the sterols required, however, a sterol with Δ-5- unsaturation is required for growth. Heme-deficient cells, did not grow on defined medium using respiratory substrates such as glycerol and ethanol. The sterol content of the cell can affect respiration (Parks, *et al.*, 1995a). Ethanol and glycerol are respiratory substrates. The sensitivity of antifungal agents that are inhibitors of sterol biosynthesis is enhanced when cultures are grown on respiratory substrates (Parks, *et al.*, 1995b).

Cytochrome P-450 isozymes are iron-containing hemoprotein which catalyze the monooxygenation of a broad spectrum of lipophilic substances, such as fatty acids, sterols, sex steroids, glucocorticoids, mineralocorticoids, vitamin D, leukotrienes, prostaglandins and retinoic acid. A lipophilic compound can bind tightly to the apoprotein of cytochrome P-450 (Cauwenbergh, *et al.*, 1989; Aoyama, *et al.*, 1991). Therefore, lipophilic compounds may influence P450 enzymes. Coumarin or 4-methylcoumarin are 3-hydroxylated by rat-liver microsomes and 4-methylcoumarin given orally has induced microsomes (Feuer, 1970).

Inhibition of one of the enzymatic steps in the metabolic pathway can cause accumulation of substances before that step. This is the case for azoles, which inhibit 14-demethylase in ergosterol biosynthesis. In the case of test compounds no sign of squalene or lansoterol accumulation was observed in the angelicin and compound 61 treated *C. albicans*. Azole antifungals are shown to induce hepatic CY P450 isozymes (Ronis, *et al.*, 1994). As coumarins are substrates for a variety of P450 enzymes (Ito, *et al.*, 1994), it could be possible that in a way they lead to induction of this type of enzymes. In fact, it has been reported that coumarin and some compounds with electrophilic centres induce phase II

enzymes (like glutathione-S-transferase, GST) which participate in the reaction for detoxification of carcinogens (Talalay, 1992). If this theory is true, the rise in ergosterol level after treatment with coumarins can be easily explained. In addition, the reason for antagonism of coumarins with azoles can be understood. However, an increased level of ergosterol *per se* could be enough to reduce the inhibitory activity of azoles, which depend on depleting the ergosterol pool in the fungal cell (Ghannoum, *et al.*, 1994; Parks *et al.*, 1995).

Other factors could be involved in the rise of ergosterol in fungal cells. One is pH, since lanosterol production is shown to be favored at the optimum pH levels. The other factor is sulphur metabolism as cysteine acted as an elicitor at lower concentrations tested (0.9 mM) for several triterpenes and ergosterol. Allicin, and methionine elicited production of the C-21 aldehyde derivative. Cysteine is not inhibitory to sterol biosynthesis; in contrast it has a stimulatory effect on ergosterol production. This suggests that cysteine plays some role in sterol biosynthesis, from lanosterol to ergosterol, via certain intermediates including the alkylation (C-24) of the side chain by S-adenosyl methionine. Ergosterol is also affected by variables such as fungal species, O_2 availability, temperature, growth substrate, and the method of analysis used (Kahlos, *et al.*, 1994). Up to this study, there has been no method available for producing only one major triterpene or sterol as the chief product of fungal metabolism. However, coumarins, like sulphur compounds, may be noteworthy elicitors at specific concentrations.

Agents like db-cAMP, forskolin (adenylyl cyclase activator) and IBMX have been shown to enhance both basal and phenobarbital-induced COH (coumarin 7-hydroxylase) activities and CYP2A5 mRNA levels indicating that cAMP plays a major role in CYP2A5 expression. Inhibitors of PKc (protein kinase C) like staurosporine, tyrosine kinases, like genistein and lavendustin A, PKc inhibitor, phorbol acetate and a generator of nitric oxide, sodium nitrate, did not affect COH basal activity or inducibility (Salonpaa, *et al.*, 1994). Regarding the observed inhibitory activity of coumarins, this cAMP-mediated enzyme induction could be considered one of the strongest explanations for the rise in ergosterol level.

In addition to what mentioned above, the level of ergosterol and its biosynthesis, as for any other biomolecule, should have regulatory systems. Although this system has not been proven to date, there are studies suggesting ergosterol may function in wild type yeast as a feedback regulator of sterol biosynthesis (Lorenz, *et al.*, 1991; Venkatramesh, *et al.*, 1996; Passi, *et al.*, 1994; Pinto, *et al.*, 1985; Koller, 1992). Therefore, regardless of the involvement of cAMP in such regulation, coumarins may be disrupting the feedback control of ergosterol biosynthesis.

The antagonism of PDIs and azole antifungals or AmB can be explained by the increased biosynthesis of ergosterol. According to previous studies, short time exposure of aminophylline to *Microsporum gypseum* cells resulted in decreased lipid synthesis and activity of glycerol-3-phosphate acyltransferase, while cultures grown with aminophylline exhibited increased content and synthesis of lipids due to the rise in intracellular cAMP levels (Bindra, *et al.*, 1993a). It has been noticed that lipids biosynthesis is quite interrelated in fungi, such that inhibition of ergosterol synthesis can increase the free fatty acids in the fungal cell (Ragsdale, 1975).

The role of cAMP in phosphatidylcholine biosynthesis was determined by the uptake of precursors in aminophylline and atropine grown cells. Incorporation of methionine in total lipids of *Microsporum gypseum* cells was higher in the presence of aminophylline. Aminophylline (4 mM) led to a four-fold increase and atropine (2 mM) led to a 2.3 fold decrease in cAMP levels in *M. gypseum*. Methylation of lipids is shown to be affected by cAMP (Bindra, *et al.*, 1993b). In our experiments the PDIs were co-ineubated for 24-48 h with the fungal cells and the antifungal agents. The unexpected results in some cases may have been due to the length of exposure of the cells to the PDI. Also the various responses achieved by using different phosphodiesterase inhibitors can be due to different isozymes that interact differently with the inhibitors (Essayan, *et al.*, 1994).

In addition to ergosterol biosynthesis, interaction of PDIs and azoles can be at the enzymic level too. For example, imidazoles are shown to be phosphodiesterase activators

(Walland, 1977). This might also explain the reason that antifungal azoles were antagonized by PDIs.

The reduced adenylate cyclase activity lowers cAMP levels and this can decrease the ergosterol level. In *Saccharomyces cerevisiae* inhibition of AC has reduced cell division in the G1 phase (Ilda, 1988). Considering the vital roles of ergosterol for fungal cell, a reduced cell division can be expected.

PD Inhibition by Coumarins

The coumarins synthesized showed very good PD inhibitory activity. The activities of the novel derivatives have been reported for the first time. Certain esters of dihydropyranocoumarin, dihydrofuranocoumarin alcohols and isocoumarins have previously been shown to inhibit the cAMP-phosphodiesterase from bovine heart. These naturally occurring coumarins also inhibit the high affinity cAMP-phosphodiesterase from human platelets with activities that closely correlate with those obtained using phosphodiesterase from bovine heart tissue (Thastrup, *et al.*, 1985; Furutani, *et al.*, 1975).

The circulatory effects of coumarins have been studied on the basis of the PD inhibitory activity. The reports indicate the involvement of cAMP-phosphodiesterase inhibition in coronary vasodilatory effects of acyloxydihydropyrano- and acyloxydihydrofurano-coumarins (Thastrup, *et al.*, 1983). Inhibition of platelet aggregation also occurred by increasing intraplatelet cAMP concentration due to the application of coumarins (Tawata, *et al.*, 1990; Teng, *et al.*, 1994).

3-Arylcoumarin derivatives, from *Glycyrrhiza radix*, which is a crude drug of kampo herbal medicines, have been shown to inhibit platelet aggregation, phosphorylation of 40K and 20K dalton proteins, inositol 1,4,5-trisphosphate production, intraplatelet calcium increase and phosphodiesterase activity *in vitro*. (Tawata, *et al.*, 1990)

Coumarins have other related activities on the circulatory system as well. For example, AD6 is a coumarin derivative which is able to inhibit platelet aggregation and release due to various agonists as adrenaline, PAF (platelet activating factor), Ca^{2+} ionophore and others. It has been demonstrated that this compound reduces the production of free arachidonate and diglyceride from human platelets pulse-labeled with radioactive arachidonic acid thus suggesting a possible interference with the activity of phospholipase A2 and/or phospholipase C (Porcellati, *et al.*, 1990). Phosphodiesterase inhibitors can increase immunity and viral resistance of the body. Forskolin (FK), a reversible activator of adenylate cyclase, markedly enhanced the expression of the interleukin-2 receptor (IL-2-R) on a human natural killer (NK)-like cell line, YT. The FK-induced increase in IL-2-R on YT cells was closely correlated with an increase in intracellular cyclic AMP (cAMP) level, and was mimicked by dibutyryl cyclic AMP (dbcAMP). These results suggest the possibility that the stimulation of adenylate cyclase may serve as a pathway leading to activation of the IL-2-R gene in certain types of lymphocytes (Narumiya, *et al.*, 1987). These examples show that coumarins are potent biomolecules, which can have antifungal as well as cardiac, circulatory, and immunomodulating effects.

Common furocoumarins like, 8-MOP (methoxypsoralene), psoralen, and 5-MOP were tested together with UV light at 365 nm on macrophage cells. They inhibited the enzyme phosphodiesterase in a dose dependent manner. Combination was additive rather than synergistic. 8-MOP was the strongest in this respect (Roemer, *et al.*, 1983). In another study, psoralen, 8-MOP, 4,5',8-trimethylpsoralene, (TMP), 4,6,4'-trimethylangelicin (TMA) and khellin were tested for PD inhibitory activity. TMA and TMP, which are more lipophilic than angelicin and psoralen, showed significant activity (Bovalini, *et al.*, 1987). Licoarylcoumarin was identified as strong inhibitors of adenosine 3', 5'-cyclic monophosphate (cAMP) phosphodiesterase. The structure-activity relationships of many 4-arylcoumarins were studied. In this group, 5,7-dihydroxy derivatives were generally highly inhibitory towards cAMP phosphodiesterase (Kusano, *et al.*, 1991). The above-mentioned reports support our findings that lipophilicity and phenolic hydroxy are two important factors in the PD inhibitory activity of coumarins.

The antiallergic drug disodium cromoglycate and its non-antiallergic analogue dicoumarol were found to inhibit the enzymatic activity of phosphodiesterase, respectively. It seems that the dichromone group is also one important structural feature for this inhibition (Ochoa de Aspuru, *et al.*, 1996).

The effect of AD6 (8-monochloro-3-β-diethylamino-ethyl-4-methyl-7-ethoxycarbonylmethoxy coumarin), an inhibitor of platelet aggregation, on cyclic nucleotide metabolism was investigated. AD6 inhibited selectively human platelet cyclic GMP phosphodiesterase (Hakim, *et al.*, 1988). Phosphodiesterases are at present grouped into at least seven families (Hichami, *et al.*, 1995). Therefore, if there is no clear relationship between coumarin antifungal activity and its phosphodiesterase activity, it might be due to differences in the mammalian and fungal enzymes.

Coumarins in addition to being PD inhibitors are shown to be uncouplers of oxidative-phosphorylation (Kono, *et al.*, 1977). This means that PD inhibition and lack of ATP may have opposite effects on the cAMP level of fungal cell.

In ion toxicity tests, although the MIC values are not clearly indicative of any big change, the number of colonies and growth level in each colony showed a significant decrease together with higher levels of Ca^{2+} and Na^{+} for increasing concentrations of AmB, angelicin (not so strong for Na^{+}), and DMSO. This is a sign of membrane perturbance. Altered sensitivities to NaCl and $CaCl_2$ suggest a change in ion permeability as a result of the alteration of the membrane sterol composition. Yeasts have various mechanisms which allow them to grow with high Ca^{2+} concentrations. For example, vacuoles possess a potent Ca^{2+} -uptake system driven by an H^{+}-ATPase and serve as an intracellular store for Ca^{2+} (Taguchi, *et al.*, 1994). Therefore, an increased sensitivity to this ion can be indicative of inhibited vacuolar H^{+}-ATPase or large-scale membrane damage leading to high Ca^{2+} influx.

A mutant of *S. cerevisiae* which has a disrupted ERG3 gene coding for sterol C-5 desaturase, an enzyme of the ergosterol biosynthetic pathway, has been identified. This mutant is more sensitive to high Ca^{2+} of the medium. Membrane ergosterol presumably serves as a nonspecific barrier against various chemicals in the environment (Hemmi, *et al.*, 1995).

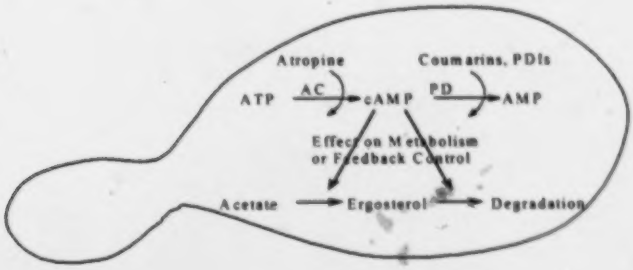


Figure 6-56. Putative mechanism of the inhibition of PD by coumarins in fungal cell and consequent events. Possible role of cAMP in ergosterol metabolism (induction of biosynthesis, or inhibition of catabolism) or interference with regulatory systems has been proposed (see the text).

The Δsyr1 mutants, which have a mutation in the gene responsible for an enzyme of the ergosterol biosynthesis pathway, were sensitive to a wide variety of drugs, chemicals, and ions suggesting that yeast ergosterol is important as a permeability barrier against various chemical stresses (Hemmi, *et al.*, 1995)

Among the natural products, as mentioned earlier, saponins, polyenes, and triterpene-glycosides cause leakage of metabolites (Lyr, 1977). Pre-incubation of *C. albicans* suspension cultures with ergosterol or cycloiridals inhibited Pi leakage when cells were subjected to saponins (Leconte, *et al.*, 1997). It seems that ergosterol should play a protective role against penetration and leakage of ions to and from the cell. However, this condition is met for comparison of ergosterol deficient with normal membrane, not a membrane with more than needed ergosterol. From our study, it seems that extra ergosterol can be as harmful as low levels of it, at least in terms of ion permeability.

Effect on H^+-ATPase:

The fungal plasma membrane proton-ATPase is an integral membrane protein that belongs to the P-type class of ion translocating ATPases. Like other P-type ATPases, proton-ATPases form an acyl-phosphate intermediate during their reaction cycle and are sensitive to micromolar concentrations of the inhibitors vanadate and diethylstilbestrol. P-type ATPases seem highly asymmetric with nearly 70% of the mass exposed in the cytoplasm and about 5% exposed to the extra-cytoplasmic compartment. These enzymes, apart from transcriptional regulation, have their activity autoregulated through generation of membrane potential. *Candida albicans* has an alkaline intracellular pH during germ tube formation, implicating the involvement of proton pumping ATPase in pH-dependent morphogenesis. P-type ATPases from both mammalian and plant sources show considerable cell surface divergence from the fungal system in the *PMA*1 (the gene encoding proton pumping ATPase) region, while those of fungi are highly conserved. Several transmembrane segments of fungal plasma membrane H-ATPase are related to those of ouabain binding sites of the gastric H^+, K^+-ATPase and the Na^+, K^+-ATPase (Monk, *et al.*, 1994).

Viable mutations within *PMA*1 cannot render the enzyme functionally inactive. Mutation in this gene must either be partially active or conditionally inactive. The *pma*1 mutations conferred a wide range of cellular phenotypes including sensitivity to low pH, weak acid loading, high ionic strength media, osmotic pressure and NH_4^+ (Perlin, *et al.*, 1988).

Hygromycin B resistant *pma*1 mutants are defective (kinetically) in the plasma membrane H^+-ATPase. The wild type *Saccharomyces cerevisiae* cells, which is normally inhibited by hygromycin B at 200 μg/ml was found to be resistant to the antibiotic by the addition of 50 mM KCl to the growth medium (Perlin, *et al.*, 1988). Several ATPase inhibitors tested (DCCD, Dio-9, octylaminoester, and miconazole) induced a rapid efflux of K^+ accompanied by the stoichiometric influx of H^+ when added at pH 4.5 to intact cells incubated in the presence of glucose (Dufour, *et al.*, 1980). The fact that the medium

with potassium phosphate caused *C. albicans* cells to show resistance to compound 23 (Fig. 6-30) compared to the medium devoid of K_3PO_4, can be explained in the same way. Inhibiting H^+-ATPase by compound 23 could produce a situation such as having kinetically defective H^+-ATPase, therefore, K^+ ions can maintain cell viability. The pH sensitivity of most of the *pma*1 mutants could also be suppressed by the addition of 0.1 M KCl to the medium but not by the addition of 0.1 M NaCl, $MgCl_2$, or $CaCl_2$ (McCusker, *et al.*, 1987). The effect of NH_3 on H^+-ATPase was not studied otherwise other factors such as Ca^{2+} channel blockage by ammonium salts (Garrill, *et al.*, 1993), could be mistakenly taken into consideration. In any case, *in vitro* studies should be performed to confirm the present *in vivo* tests.

Many antifungals including ethanol show stronger activity at higher pH values. However, the compounds with ionizable functionality that are dissociated at high pH are stronger antifungals at lower pH values. Among the coumarins tested, except compound 61, none has such ionizable functionality. Therefore, pH-dependency in their activity should be related to other factors.

In fact, omeprazole (H^+-ATPase blocker at 50 μM) inhibits the growth of *S. cerevisiae* and *C. albicans* in a pH dependent manner. Glucose-dependent medium acidification is correspondingly blocked by omeprazole. The inhibitory properties of omeprazole are blocked by pre-treatment of activated drug with β-mercaptoethanol, which is consistent with the expected formation of a sulfhydryl-reactive sulfenamide derivative (Monk, *et al.*, 1995b). A previous experiment shows that omeprazole is acting on the outside of ATPase (extracellular portion of the enzyme), and it does not need to pass the cell membrane (Seto-Young, *et al.*, 1997).

Among other agents that showed synergism with angelicin and compound 23, are valinomycin and orthovanadate. Valinomycin, which is a K^+ conducting ionophore, moves K^+ ions through bilayer lipid membranes (Slayman, 1994). It forms a lipid soluble positively charged complex with K^+, which diffuses across the membranes. In case of a high concentration of K^+ inside, a net negative charge is left inside the cell, and then K^+

diffusion slows down and becomes dependent on the influx rate of H^+ (De Waard, *et al.*, 1987). This could explain the synergy of coumarins with valinomycin. Considering the coumarins tested as H^+-ATPase inhibitors, the protons diffused into the cell are not pumped out fast enough. Then K^+ leakage is continued till the appearance of toxic effects on the cell. This depletion of K^+ can decrease fermentation potency too, since phosphofructo kinase, the key enzyme of the glycolytic pathway, is activated by K^+ ions. In addition to the toxicity caused by K^+ leakage, the transport of H^+ equivalents itself as well as weak acids such as succinate into the cell, leads to intracellular acidification and further cytotoxicity (Hasuda, *et al.*, 1994).

Orthovanadate is a phosphate analogue which blocks all known phosphorylating transport ATPases. (Slayman, *et al.*, 1990). Among natural products, quercetin and biflavonoids are also active on H^+-ATPase (Goffeau, *et al.*, 1981). Vanadate is believed to bind at the site from which phosphate is released. However, vanadate insensitive H^+-ATPase has also been identified (Perlin, *et al.*, 1989). Vanadate is the most effective inhibitor *in vitro*, but needs a high concentration *in vivo* to affect fungi; this may be due to high concentration of phosphate in the medium or poor entry into the cell (Monk, *et al.*, 1994).

In this research, coumarins were found to some extent to act like ethanol. Ethanol inhibits glucose induced proton efflux in energized organisms and enhances influx of proton under de-energized conditions. Increased concentration of ethanol results in a decrease in intracellular pH, which limits fermentation rate. Plasma membrane ATPase activity is indeed inhibited by ethanol. Modification of lipid composition also influences proton influx. It is noticeable that ethanol is the only alcohol with these effects (Mishra, 1993). Methanol that was used as a solvent in our experiments did not show signs of ATPase modulation.

Assuming the plasma membrane ATPase is required to maintain the internal pH of the cell, two methods are suitable to assess the pH sensitivity of H^+-ATPase modulated fungi. The first test was based on the use of membrane-permeable weak acids as a means of acidifying the internal pH. Acetate and other weak acids have been shown to act as

growth inhibitors in yeasts as well as in other fungi. CH_3COOH can passively cross the membrane and then dissociate inside the cell to form CH_3COO^- and H^+, thus lowering the intracellular pH. In the second test of pH sensitivity, the ability of all of the fungi to grow in RPMI (without acetate) at pHs 4.0, 5.0, 6.0 and 7.0 was examined. These tests have also been used to identify mutants deficient in plasma membrane ATPase (*pma* 1) (McCusker, *et al.*, 1987). Intolerance of fungi incubated with angelicin and compound 23 to acetate and also their increased susceptibility in lower pH values support the idea of inhibition of H^+-ATPase by these compounds. It has been reported that furanocoumarins and other coumarins affect the activity of ATPase in plant cells (Podbielkowska, *et al.*, 1996). To test the theory of H^+-ATPase inhibition in fungal cells by coumarins in a more precise manner, it was decided to estimate pH values in cell compartments using NMR techniques.

As eukaryotes, yeasts possess several intracellular compartments: cytoplasm, mitochondria, vacuoles, etc. There are several proton-translocating ATPases identified in yeast: one associated with the plasma membrane, another with the inner mitochondrial membrane, and one associated with the vacuolar membrane. Therefore, yeasts possess, in principle, the means to maintain a distinct pH in each of these compartments. In particular, there is evidence that the vacuolar space, which in resting yeast cells is the largest intracellular compartment, is considerably more acidic than the cytoplasm (Gillies, *et al.*, 1982).

From the position of (chemical shift) of the phosphate peak in the ^{31}P-NMR spectra, the intracellular pH can be determined (Thevelein, *et al.*, 1987b). In order to determine the values for the intracellular pH, the chemical shift of the intracellular Pi (Pi_{int}) resonance can be monitored and be compared with a Pi titration curve obtained with RPMI medium.

The assignment of the NMR resonances was made by comparison with previously published NMR spectra. The chemical shift of the Pi_{int} resonance of cells metabolizing glucose is shifting slightly upfield in more acidic intracellular pH. The chemical shift of the vacuolar Pi resonance is also pH sensitive and gives information on the acidity of

vacuoles. This resonance is hidden under the large extracellular Pi (Pi_{ext}) resonance. Therefore, the pH of vacuole can be determined indirectly from the difference between the chemical shift of the terminal polyphosphate (PP1) resonance and that of the longer polyphosphate chain (PPn). The chemical shift of ATP β resonance is sensitive to Mg^{2+} levels, which could vary upon using different carbon sources and the physiologic condition of the cell (Lohmeier-Vogel, *et al.*, 1995a). As the position of the peak and its shape (splitting) depends also on the conformation of the molecule, none of the ATP peaks is a reliable pH indicator (Slavic, 1989). After addition of glucose to fungal cells, more ATP is being generated and ATPase should function in a more efficient way. The cytoplasmic pH of *C. tropicalis* cell suspensions metabolizing glucose is 7.8 regardless of whether the cells are oxygenated (Lohmeier-Vogel, *et al.*, 1995a).

Phosphate ester hydrolysis by itself should lead to a lower pH indicating that an increase in pH cannot be caused by polyphosphate consumption (Gillies, *et al.*, 1981). Using ^{31}P-NMR, it was shown that the levels of cytosolic phosphorous compounds are maintained constant for about four hours after shift to phosphate starvation medium, whereas the amount of polyphosphate decreased. Therefore, the dynamic of phosphate hydrolysis is not very fast and during our NMR experiment they should have not happened (Shirahama, *et al.*, 1996).

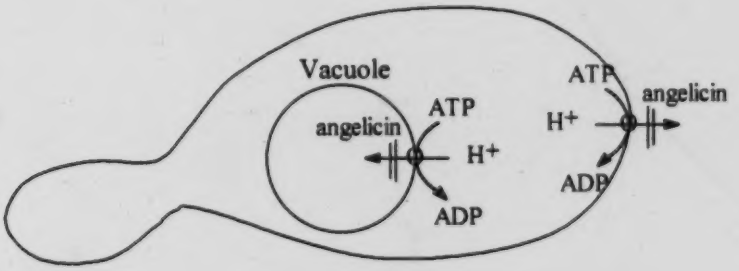


Figure 6-57. Putative mechanism for the inhibition of glucose induced medium acidification and induction of cytoplasmic pH decrease and vacuolar pH increase by coumarins mediated through H^+-ATPase inhibition.

The proposed inhibitory action of H^+-ATPase of fungi can again have more side effects in the fungal physiology. Addition of organic acids, such as acetate and butyrate, at low pH and under aerobic conditions, also caused an immediate increase in the cAMP level and an immediate drop in the intracellular pH. Azide and DNP (dinitrophenol) increase cAMP because of lowering intracellular pH not membrane depolarizing activity. In the anaerobic condition, azides, DNP and acids were much less effective in increasing cAMP level. However, addition of glucose restored their activity, since the ATP level is a limiting factor for cAMP synthesis (Thevelein, *et al.*, 1987a). Based on what was mentioned before, the increased cAMP can raise ergosterol levels. Because of the different pH optima of adenyl cyclase (pH 6) and phosphodiesterase (pH 8) a lowering of the intracellular pH is expected to raise the cAMP level (Londesborough, 1977).

The observed decrease of H^+-ATPase activity does not seem to be due to change in the sterol composition of the membrane (Cobon, *et al.*, 1973). It has been shown that the plasma membrane H^+-ATPase activation induced by decanoic acid is correlated with an alteration in membrane lipid constituents. Decanoic acid caused changes in lipid composition of the plasma membrane. In this study, ergosterol increased compared to other sterols. *S. cerevisiae* adopts two different strategies to cope with the changes caused by decanoic acid. One is activation of the enzyme and the other is modification of the membrane composition (Herve, *et al.*, 1996).

Since db-cAMP can stimulate acid secretion by canine parietal cells (Nagaya, *et al.*, 1990), it would be interesting to know if a similar action is happening in the fungal cell. If this is the case, coumarins with weak PD inhibitory activity should show stronger H^+-ATPase inhibition *in vivo*, because the cAMP level would no longer activate ATPase and the net effect is higher ATPase inhibition. UV radiation at 290 nm and 365 nm was shown to inactivate two forms of the K^+ stimulated ATPase associated with the plasma membrane of suspension cultured cells of *Rosa damascena*. Therefore, it is expected that using UV plus photodynamic coumarins should exhibit greater inhibition of ATPase (Imbrie, *et al.*, 1984).

The mechanism by which H^+-ATPase is inhibited by coumarins does not seem to be involved in –SH group blockage. As it has been indicated before, none of the Cys residues proved essential either for biogenesis of the ATPase or for activity. Likewise, ATP hydrolysis and ATP-dependent H^+ pumping were essentially normal in most of the mutants with varied Cys residues in their ATPase. It has also been shown that if a disulfide bond is formed in the yeast ATPase, it does not play an essential structural or functional role (Padmanabha, *et al.*, 1994).

H^+-ATPase can be used as a specific target for antifungal therapy. Although similar enzymes also exist in the human body, the differences in eukaryotic H^+-ATPases are large enough to allow a successful drug design, specific for one type of enzyme. For example, the similarity between *S. cerevisiae PMA*1 and other fungal *PMA*1 homologues is about 70% at the deduced amino acid sequence level (Mason, *et al.*, 1996).

Morphological changes:

Most of the pictures taken by TEM technique showed changes in vacuoles of the cells treated with coumarins. It was suggested earlier that coumarin treatment changes the pH of vacuoles and also this action might be mediated through inhibition of vacuolar H^+-ATPase. Here, we try to focus on the role of vacuolar ATPase and consequences of its inhibition.

Vacuolar, or V-type, proton translocating ATPase is distributed in bacteria, yeasts and mammalian cells. In higher Eukaryotae, these proton pumps are found in numerous intracellular organelles of both the constitutive sorting and specialized secretory pathways. In addition, these enzymes participate in urinary acidification as well as osteoclast mediated bone reabsorption. From a pathophysiologic standpoint, vacuolar proton pumps are essential to nutrient uptake and processing in the food vacuole of *Plasmodium falciparum*, and evidence suggests that V-type proton pumps may confer resistance to chemotherapeutic agents in cultured tumor cells (Crider, *et al.*, 1994).

Vacuolar ATPase is sensitive to bafilomycin A1, N-methylmaleimide, KNO_3. and resistant to oligomycin, azide and vanadate. The vacuolar ATPase utilizes the energy generated by hydrolysis of ATP to pump protons into the vacuole lumen. This results in a calculated electrochemical potential difference of protons on the order of 180 mV contributing to both a decreased pH and a membrane potential of approximately 75 mV for *S. cerevisiae*. The primary mechanism for transport of storage molecules into the vacuole appears to rely on a proton antiport system. Although the vacuolar ATPase is the major energy donor for these transport systems, there is some evidence that a pyrophosphatase activity is associated with the vacuole membrane which may be responsible for a PPi-dependent formation of a pH gradient. By altering the ion conductivity of the vacuolar membrane, proton uptake activity can be blocked or enhanced while the ATPase activity is relatively unaffected. Treatment of yeast cells with bafilomycin A1 causes an increase in vacuolar pH, which can be demonstrated by the abolition of quinacrine accumulation in the vacuole.

One of the most prominent features of eukaryotic cells is the reliance on subcellular compartmentalization. The presence of distinct membrane enclosed organelles allows the cell to spatially separate otherwise competing reactions. The various catabolic and anabolic reactions in the cell may be controlled partly by separating them from each other; as doing chemical reactions in different containers and then storing the products to different containers after reactions are completed. The vacuole is the main storage organelle for a variety of metabolically important compounds and ions. This role in storage is not a passive one. The observation that some yeast mutants, which lack a normal vacuole, are pH sensitive suggests that vacuoles may play a role in homeostasis of the intracellular pH. These mutants also show some degree of osmo-sensitivity, indicating an additional role in osmoregulation. In addition other mutants defective in vacuolar protein sorting have extremely large vacuoles, which may reflect a defect in osmo-regulatory capabilities. The membrane potential-dependent cation channel may afford some control. Since the vacuole and cytosol are isotonic, this channel may be an osmotic regulator, which acts to balance the osmotic potential differences resulting from the uptake of cations into the vacuole (Klionsky, *et al.*, 1990).

NMR has been used to identify poly P in intact cells. However, NMR detection requires high concentration and fail to measure the poly P in aggregates and in metal complexes. When the organism is stressed at alkaline pH, amines enter the vacuoles and are neutralized by protons released by the enzymatic hydrolysis of poly P. Thus poly P as a result of its hydrolysis, can provide a high capacity buffering system that sustains compartmentation of amines in vacuoles and protects the cytoplasmic pH (Kornberg, 1995). The importance of PP in the physiologic functions of the cell requires its regulation and involvement of different enzymes in anabolism and catabolism of PP (Kumble, *et al.*, 1996).

In *Saccharomyces cerevisiae* the PP usually accounts for about 1.5% (as P_2O_5) of the dry weight and for 37% of the total phosphate. The majority of the PP consists of chains with ca. 20 phosphate units; the largest molecules contain up to 260 units.
Vacuoles were found to contain large pools of amino acids, particularly basic species such as arginine. In addition, S-adenosinylmethionine and other cationic compounds may be accumulated within the vacuoles. Most of the PP of *Saccharomyces cerevisiae* is localized in vacuoles, mainly as counter ion (Urech, *et al.*, 1978).

In addition to the changes observed in TEM pictures, SEM photos show some wrinkles in the cell wall of the cells. IBMX at 1 and 10 μM concentrations caused an increase in intracellular cAMP and stimulated the production of endoglucanase (Sestak, *et al.*, 1993). A change in the sterol composition of the membrane is shown to lower overall specific activity of the enzyme, chitin synthase (Walker-Caprioglio, *et al.*, 1990). cAMP has important roles in fungal physiology, such as yeast to hyphae transition (Egidy, *et al.*, 1990; Larsen, *et al.*, 1974; Nimi, *et al.*,1980; Cho, *et al.*, 1992). Also, cAMP can induce translation of some genes and can affect the cell cycle in fungi, and vertebrates (Lane, *et al.*, 1997).

cAMP in *C. albicans* rises during yeast to mycelial form transition, even at lower temperatures. Cysteine, which suppressed germination, also reversed the increase in

intracellular cAMP (possibly through activation of cyclic nucleotide phosphodiesterase as in *E. coli*). Cysteine promotes yeast like growth via, for example, reduction of disulfide linkages in glucomannan protein. In *M. rouxii* the addition of db-cAMP or cAMP induced the yeast like morphology. *Mucor racemosus* is similar to *M. rouxii*, however, *Histoplasma capsulatum*, like *C. albicans* tends to be in a mycelial form in this condition (Nimi, *et al.*, 1980). Although no mycelial form was observed in our study, the increased cAMP could be responsible for some of the morphologic changes seen in the SEM study.

The *Candida* cells treated with compounds 61 and 23 clearly show signs of multibudding. Each *Candida* yeast cell can normally have a budding scar or the daughter cell still attached to it (Yoshii, *et al.*, 1976). In an interesting study, *pma*1-mutants showed multiple budding (McCusker, *et al.*, 1987). This increases the possibility that inhibition of H^+-ATPase is responsible for the multibud structures.

The physiologic roles of ergosterol have not been studied in detail. However, it is known that it can, for example, stimulate cell proliferation and polyphosphoinositide metabolism in *Saccharomyces cerevisiae* (Dahl, *et al.*, 1985). This may explain the multibudding of *C. albicans* incubated with coumarins. A sterol with special structural features is required at hormonal level (10 nM) to allow cell cycle completion (Lees, *et al.*, 1995). The other very possible cause of the multibudding phenomenon could be PD inhibition. A similar observation was done in treatment with caffeine, which at the subinhibitory concentrations caused an increase in unusual modes of proliferation with signs of multiple budding in *Candida albicans* (Mittag, 1994).

Although there are reports of interaction of coumarins with mitochondria (Zboril, *et al.*, 1973), no morphological changes in this organelle is evident from TEM pictures.

The fungi treated with fluconazole show deformities and vast damages in the cell membrane. They also may lack a vacuolar compartment and accumulated small vesicles and a variety of abnormal membranous organelles within their cytoplasm. The precise

origin or function of these structures is not known. They may represent remnants of a vacuolar compartment.

In summary, an increase in the ergosterol level is a novel observation. Although, in some cases, it has been reported that ergosterol can be increase compared to other sterols present in the cell, in all of the studies, the reported increase of ergosterol was infact a "less decrease" as compared to general decrease in sterol levels. However, here we reported an increase of ergosterol on its own based on the cell dry weight. Our findings suggest that this increase is due to the PD inhibitory activity of coumarins. Phosphodiesterase inhibitors like pentoxifylline, IBMX, and theophylline, either reduce or antagonize the effect of azole antifungals. This supports the idea of a relationship between cAMP and ergosterol levels, as it has been shown that PDIs can affect fatty acid biosynthesis and composition in fungal cell (Buttke, *et al.*, 1988; Boll *et al.*, 1980). It is possible that an increased ergosterol level in *C. albicans* followed by coumarin treatment is a consequence of increased biosynthesis of ergosterol as a result of induced enzyme activity, availability of precursors, or both.

This finding can have significant implications in antifungal research. Clinical consequences might emerge from our findings are as follows: Antifungal compounds especially azole derivatives could be considered to show reduced activity while being used with phosphodiesterase inhibitors like theophylline (drug-drug interaction). A clinical potentiation of activity may occur in concomitant usage of azoles and atropine analogues (combination therapy).

Fungal H^+-ATPase is a novel target in antifungal therapy. Inhibition of the H^+-ATPase has been fungicide in action (Monk, *et al.*, 1995a), since it has a crucial physiologic function to play. It was noticed that unlike many other fungal targets, such as ergosterol, which mutants lacking them can survive, there is no mutant totally lacking this target. There are specific inhibitors of each types of H^+-ATPase, for example bafilomycin A1 is an inhibitor of the vacuolar type H^+-ATPase (Galvin, *et al.*, 1996). These agents can not be considered as drug candidates, since they mostly have large structures with possible

allergenic property, or they are active only on the enzyme *in vitro*. Therefore, by considering safety of coumarins for human use (Keightley, *et al.*, 1996), looking at them as H^+-ATPase inhibitor candidates is promising.

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Chapter 7

General Conclusions and Summary

With the employment of modern chemotherapeutic modalities in the 1960's, susceptible hosts were created for several opportunistic organisms and with the advent of the AIDS era in the 1980's, a broad range of opportunistic pathogenic fungi are making their appearance on the medical scene. The role of commensals, such as the *Candida* species, is changing. Ranging from a 75% increase in small hospitals to over 400% increase in some large care centers, *Candida* is now ranked as the third most common causative agent of nosocomial blood stream infections in most hospitals (Pfaller, *et.al.*, 1992; BSACWP, 1992).

Research is going on in many aspects of fungal pathogenesis and treatment. The development of azoles has revolutionized the treatment of many fungal infections, but still treatment of many of them necessitates application of the highly toxic drug, amphotericin B or a combination of drugs. Emergence of new resistant species of fungi in addition to the poor safety and pharmacokinetic profile challenges clinicians in their approach to treat the fungal infections. Combination therapy, improving delivery systems, immunotherapy, synthesis of new analogues of current antifungal drugs, and finally discovery of new prototypes are among the possible methods of improving antifungal therapy in general.

In the field of drug discovery there are two main branches which are pushing the limits. One is computer aided drug design and the other is natural product-based research. Although over many centuries man kind has heavily relied on nature for food, drug and other needs, in the recent years this dependence has been under constant attack from man-made products. The success or failure in this matter, or even the legitimacy of this approach has been questioned. Our increasing knowledge in different fields from subatomic forces to behavior of large molecules in delicate interactions has allowed us able to refine our tools of designing new drugs. However, the vast majority of unknowns leave these efforts futile in many cases. Invention of tailor-made drugs are not possible

before great progress in other branches of science. Waiting for development of mathematics, computer science, crystallography of enzymes, and gene function studies to help drug discovery could jeopardize the health of many innocent patients over time. Now, there is the other option, natural resources. From one side nature has generously provided us with over 25% of the present drugs directly, and more than 60% of whole drugs indirectly (Farnsworth, 1996; Cragg, *et.al.*, 1997). From the other side, natural products, in particular, higher plants have been under great ecological pressure from pollution, logging, deforestation and so on, which has increased the likelihood of losing their genetic pool as they become extinct.

In this thesis, therefore, we have focused on understanding the potential of natural products and higher plants in particular. The approach that was described in chapter one for selecting plants is unique. There are several studies considering traditional medicine as their main factor of plant selection (McCutcheon, *et.al.*, 1994; Brito, *et.al.*, 1996; Oliver-Bever, 1983; Wang *et.al.*, 1995), or chemotaxonomy as a mean of justifying their choice (Hoffman, *et.al.*,1993). However, we tried to consider a group of eight factors including traditional medicine and taxonomy in our selection criteria. This work was started on 40 plants from 16 different families.

The total ratio of active extracts on at least one fungal species was found to be 65%. This number compared to previous reports of similar screening for antifungal activity is considered to be very high. Although the type of selection factors and preliminary study on each choice to be entered in our survey was certainly playing an important role, other factors might affect comparability of different reports. The factors that affect the nature of plant extracts, from growth site and condition to processing and further study for bioactivity could change the results. For example, we found out that the antifungal susceptibility method could be an important factor, which is ignored in many studies, mainly due to their different sensitivities (Table 7-1).

Table 7-1. Sensitivity of test method based on MIC of fluconazole on *Cryptococcus neoformans* KF-33.

Method	MIC (μg/ml)
Microwell broth dilution	0.195
Agar dilution	2.5
Disk diffusion	20
TLC agar overlay	>200

In addition to the method, the fungal strain and culture medium can affect the results of antifungal screening tests. Some of the culture media are more suitable for certain purposes. For example we found that on a certain group of plants there is more incidence of inhibition when using PYEA followed by SDA and finally PDA. In case of growth stimulation a reverse trend was observed. Therefore we tried to use SDA as a standard medium capable of showing both growth stimulation and inhibition to an acceptable level.

Growth stimulation of fungi by plant extracts was an interesting observation. This phenomenon is usually ignored all together. It was shown that many extracts with low or no antifungal activity, in fact show growth stimulation. This apparent antagonism might be the origin of many false negative reports and controversy. A plant might have both antifungal compounds and fungal growth factors. Depending on the environmental, genetic and ecological factors, the ratio of these two may well be in favor of only one; therefore the final outcome could be simply determined according to the existing level of each bioactive group of compounds.

These results have the potential for further research in two different ways. First, the fungal growth stimulatory compounds can be isolated. After structure elucidation and sufficient literature search, the mechanism of stimulation could be determined. Accordingly, proper antagonists can be designed and synthesized to reverse the stimulatory effect to inhibition. Of course, as a health care professional the fungal invasion has to be stopped, but as a fermentation biologist, induction and maintenance of

rapid fungal growth is a desired effect. Second, the exact ecological and physicochemical conditions required to favor the production of a set of bioactive compounds can be studied. The results of such a study will, in turn, translate into modification of conditions so that one group of compounds dominantly would be produced in the plant or cell culture. A deeper study could certainly lead to identification of genes necessary for biosynthesis of the desired compounds and subsequent genetic transformations are predicted. This process has been exemplified in a more detailed fashion somewhere else (Jiang, 1995).

In a continuation of lead discovery, it was attempted to purify active constituents of one of the plants showing more promising antifungal activity. In this respect *Diplotaenia damavandica* was chosen. The selection was performed mostly based on the spectrum of activity, potency, lack or low toxicity, and rarity of the plant. The rarity is important to prevent possible redundancy of the outcome.

Bioassay guided isolation of active principles was carried out on *D. damavandica*. Four coumarins namely, angelicin, libanorin, psoralen and auraptene (meranzin) were purified and their structure elucidated. Among these, angelicin had the best profile of antifungal activity and was selected as our lead structure for further modifications.
Although the antifungal activity of these type of natural coumarins has been known, all the reports are indicative of application of UV radiation as the co-requisite for this property (Juttermann, *et.al.*, 1985; Martin, *et.al.*, 1966; Weimarck et.al., 1980; van der Sluis, *et.al.*, 1981). Our findings suggest that in the absence of UV radiation the antifungal activity is maintained. This is particularly important for the angular furocoumarins such as angelicin, which lack DNA cross linking activity, therefore indicative of other mode of actions.

Traditional usage of plants has been a guiding tool for finding new biomolecules in plants. In addition, the success rate of such an approach has been reported high (Brito, 1996). Therefore, it was intended to investigate the modern basis of one of the most popular native Indian traditional medicines, *Fraxinus americana* (Krochmal, *et.al.*, 1984;

Kruedener, *et.al.*, 1995; Densmore, 1974). There are many different traditional uses mentioned for this plant. For example, tonic, snakebite healing, sores, and aphrodisiac are among the main ones. However, the activities that are close to the focus of this research are sore and snakebite treatment. These are mostly external applications and could be involved in complications like infection.

Although the total extract of the bark of *Fraxinus americana* did not show antifungal property, this did not stop us from further phytochemical study. The reason was mostly the potential facing of fungal growth stimulant in the extract. This was partly true as one of the major components isolated from this plant, namely ligstroside, showed weak antifungal activity, while the extract was originally inactive. However, it is not known to what extent this activity could participate in the final traditional usage in skin diseases.

Study of the isolated components of *F. americana* bark on other relevant targets were done as well. One of the processes involved in skin diseases is inflammation. In this biologic reaction, involvement of many tissues and enzymes like lymphocytes and lipoxygenase has been proven (Negro, *et.al.*, 1997; Cashman, 1996; Drazen, 1997; Wenzel, 1997; Brady, *et.al.*, 1996). Verbascoside showed to be a potent inhibitor of lipoxygenase. The inhibitory action of verbascoside on 5-lipoxygenase can be a good example of modern explanation of traditional usage of herbs and other natural sources.

The next step in a typical drug development process is lead optimization. This topic is of course very vast and pharmaceutical companies set aside large portions of their R&D units to this area. The main purposes of structural modifications are improvement of physico-chemical properties of the molecule and change of the biological activity for a better profile of action. Lead modification can include simplification of the structure to determine the most necessary parts for the desired bioactivity. It can also cover a systematic SAR (structure-activity relationship) or QSAR (quantitative structure-activity relationship) to understand the electronic and special requirements of the active molecules. Other type of lead modification is a process of grafting different pharmacophoric groups to a mother structure.

To study the simplest form of angelicin structure necessary for the activity, different building blocks were studied. However, it became evident that simple forms are not as active. Therefore, presence of oxygen and/or alkyl substitution was apparently important. Different arrangement of rings relative to each other and furan-ring oxygen atom did not provide a definite superiority. Therefore among the structures that was studied, angelicin continues to be one of the best for antifungal activity.

Other modifications were started from the 7-hydroxy coumarin derivatives. It was shown that a free 6-OH is needed for a better antifungal profile. However, a more non-polar group at position 8 of simple coumarins can improve the activity as well.

Since mother nature is a big teacher, it was tried to look at some strategies used in natural antifungal and apply them on coumarins. One of these was connecting amino acids to the molecule with the hope of increasing the transport of the molecule into the fungal cell. Three different amino acids, leucine, phenylalanine, and lysine in protected and unprotected forms were coupled to the furanocoumarin nucleus. Unfortunately, this attempt was not successful, except for the phenylalanine derivative **34**, which showed moderate activity.

The practice of adding different fragments that are found in antifungal molecules is common for coumarins. Examples in this regard are morpholine derivatives (Gursoy, *et.al.*, 1996; Geethanjali, *et.al.*, 1995; Nofal, *et.al.*, 1990) imidazole derivatives (Gudasi, *et.al.*, 1994; Hammad, *et.al.*, 1990) and other azole derivatives (Hosmani, *et.al.*, 1996; Nofal, *et.al.*, 1990). Addition of parts of antifungal groups to the coumarin moiety was also tried for this project. In this respect, acetylenic antifungals (Grayer, *et.al.*, 1994, Harborne, 1978), and long chain hydrocarbon antifungals with different functionalities (Gershon, *et.al.*, 1979, 1978; Parang, *et.al.*, 1996) were the major ones that were considered.

Synthesis of several derivatives in each group of the above mentioned pharmacophores was carried out. The successful candidates were compounds **23** (a 7-hydroxycoumarin derivative), and **38** (a furocoumarin derivatized at 2'). Although it was mentioned earlier that for simple coumarin derivatives, a free 6-hydroxyl group is important for antifungal bioactivity; for the long chain furocoumarin derivatives hydrophobicity was found to be a critical factor. It was noted that there is a maximum bioactivity at the optimum log *D* value of about 5.

The study of mode of action of this type of antifungals is important from different points of view. In an academic field of work, the possibility of finding new targets and new modes of action as a result of following your own curiosity is satisfactory for the scientific community. However, the more important impact would be to the applied side of drug discovery program by pharmaceutical companies. Nowadays, we read about high throughput screening and combinatorial chemistry. Although we might increase the speed or the sample number, but this is usually at the cost of deeper and more basic studies, which look at the problems through designed experiments via wise guesses and observations. An example is the largest biological screening of plants ever performed, which was by NCI, covering only anticancer activity of about 50,000 species from 1957-1991 (Kinghorn, *et al.*, 1993). Interestingly, the outcome was so little and so many false negatives were reported afterwards, that questioned the set up of such screenings.

Application of studies of mechanism of action can help improving the screening process. For example, we found that MOPS buffer can interact with coumarin antifungals as well as other standard antifungals like AmB. This interaction was found for other morpholine derivatives that are similar to MOPS. Now, in a pharmaceutical company that are using this buffer routinely for their screening of synthetic or naturally derived antifungals, this interaction can be the origin of many false negative in otherwise positive reports. It would be interesting to know that MOPS buffer was originally recommended to avoid from adverse effect of other buffers on the stability of AmB in the culture medium.

Most of cell components considered being targets in the fungal cell are the result of finding a bioactive molecule acting on them. Therefore, many scientists look for the well known targets; the direction is dictated by the main stream. A simple question like, "how is ergosterol metabolized in the fungal cell?" is often left orphan. In this study, it was found that ergosterol level of fungal cell was increased following the administration of cAMP analogues or coumarin compounds. This was pinpointed when the author was looking for the effect of different cellular messengers on the dimorphism in *Candida albicans* (Sardari, *et.al.*, 1997). Following this finding, it was possible to investigate the interaction of cAMP and coumarins from one side and the ergosterol-affecting antifungals from the other side. It then became clear that the outcome of these interactions all support the idea of involvement of cAMP in ergosterol level in fungal cell. Since we want to overcome fungal cell at this particular project, it was very interesting to see how we can use this observation. Assuming that ergosterol is necessary for most fungal organisms, and at the same time its reduction by azole-type drugs can be lethal to the fungus, why not take advantage of the phenomenon described earlier. Therefore, by reducing the cAMP level of the cell, we should be able to decrease ergosterol in the cell. This was a memorable moment for the author to see that atropine, which can block adenylate cyclase, is synergistic to both azoles and AmB. This is a reward to a basic curiosity.

Needs are said to be mother of inventions; many accidents have also been mother of discoveries. The next important finding came as a result of testing antifungal activity of coumarins in old media. It was noticed that the MIC values are higher in fresh media than in older ones. Absorbing atmospheric CO_2 by weakly buffered media makes them slightly acidic and this could be the reason why the MIC values were different. Another fine observation was the difference in color of the media containing angelicin compared to other antifungals after incubation. RPMI medium that contains phenol red is weakly buffered and shows changes of pH due to acid production by the fungi. This was a mystery until the first portion of the problem (effect of pH on MIC) was solved by persistent curiosity.

^{31}P-NMR technique is clearly indicative of change in intracellular pH (acidification) of fungi treated with angelicin and compound 23. In addition, reduced acid production in both static and glucose induced conditions were observed in the treated fungi. These observations together with other supportive facts like synergism of coumarins with ATPase inhibitory agents led us to conclude that the test coumarins are inhibitory to H^+-ATPase or proton pump of the fungal cells examined in this section.

Morphological studies were considered to be supportive of biochemical studies. Lack of a clearly visible cell membrane in TEM pictures of *Candida albicans* cells treated with angelicin can be related to an ergosterol increase in the cell. The extensive deformities and/or damage observed in the vacuole of the coumarin-treated cells could be a result of inhibition of V-type proton pumps located in the vacuolar membrane.

At the end, the author wishes to see that as about 80% of the world's population rely on natural sources and traditional medicine for treatment of diseases (Farnsworth, *et al.*, 1985), more resources would be allocated for studying the mysteries in this field and more scientists participate in these projects before it would be too late.

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